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Departamento de Química

Rita Daniela Marques Sousa

Acidificação de resíduos industriais para obter substratos microbianos

Industrial byproducts acidification to produce microbial substrates

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Dissertation submitted to the University of Aveiro to meet the requirements for the Degree of Master in Biotechnology, specialization in Industrial and Environmental Biotechnology, performed under the scientific guidance of Prof. Luísa Serafim, Assistant Professor at Department of Chemistry, University of Aveiro, and Prof. Ana Xavier, Assistant Professor at Department of Chemistry, University of Aveiro.

Obstacles are the raw material of great accomplishment.

the jury

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acknowlegments

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Thanks to all of you.

palavras-chave Ácidos orgânicos de cadeia curta, fermentação acidogénica, culturas microbianas mistas, licor de cozimento ao sulfito ácido.

resumo

O uso excessivo dos recursos fósseis está na origem da introdução de processos mais sustentáveis, que usam fontes renováveis como matéria prima, como a biomassa. Devido à sua abundância, baixo custo e vasta disponibilidade, a biomassa lenhocelulósica apresenta-se como um substrato promissor para a produção de químicos e energia, entre outros. Os ácidos orgânicos de cadeia curta (SCOA) apresentam um enorme potencial devido à sua ampla aplicabilidade e ao facto de poderem ser produzidos biologicamente, por fermentação acidogénica (AF), a partir de fontes renováveis, como é o caso do licor de cozimento ao sulfito ácido (HSSL). A otimização e controlo deste processo é crucial e inclui a monitorização de parâmetros como a temperatura, pH, taxa de carga orgânica, tempo de retenção, origem do inóculo e conformação do reator.

No presente trabalho, a AF do HSSL foi avaliada num reator contínuo com mistura perfeita (CSTR), sem (CSTR1) e com controlo de pH (CSTR2). Para o CSTR1, dois tempos de retenção foram testados, 2,34 e 3 dias, tendo sido obtidas as concentrações médias de 3,10 e 3,53 gCOD/L de SCOA. Para o CSTR2, foram testados os valores de pH 6, 7 e 8, tendo sidas obtidas as concentrações médias de 2,36, 2,38 e 2,27 gCOD/L de SCOA. Por último, foi também testado um reator de biofilme de leito móvel (MBBR), tendo sido obtida uma concentração média de 2,71 gCOD/L de SCOA. De uma forma geral, os SCOA maioritariamente produzidos foram os ácidos acético, propiónico e butírico. Os testes batch realizados, juntamente com o CSTR2, permitiram ainda concluir que o pH tem uma influência decisiva nos perfis de SCOA obtidos.

keywords Short-chain organic acids, acidogenic fermentation, mixed microbial cultures, hardwood sulfite spent liquor.

abstract The excessively use of fossil fuels led to the necessity of more sustainable processes using renewable resources, such as biomass. Due to its abundance, low cost and broad availability, lignocellulosic biomass is a promising substrate for the production of chemicals and energy, among others. Short-chain organic acids (SCOA) have a great potential not only due to their wide applicability, but also to the fact that they can be produced biologically through acidogenic fermentation (AF) from renewable resources, such hardwood sulfite spent liquor (HSSL). The optimization and control of this process is crucial and comprises the monitoring of parameters such as temperature, pH, organic loading rate, sludge and hydraulic retention times, origin of the inoculum and reactor type.

In the present study, the AF of HSSL was evaluated in a continuous stirred tank reactor (CSTR), without (CSTR1) and with pH control (CSTR2). For CSTR1, two retention times were tested, 2.34 and 3.01 days, being the average SCOA concentrations of 3.10 and 3.53 gCOD/L achieved. For CSTR2, three pH values were tested, pH 6, 7 and 8. The average SCOA concentrations achieved were 2.36, 2.38 and 2.27 gCOD/L, respectively. Lastly, a moving bed biofilm reactor (MBBR) was also tested and an average concentration of 2.71 gCOD/L of SCOA was obtained. Generally, the main SCOA produced were acetic, propionic and butyric acids. The batch experiments performed, plus the results from all the reactors, allowed to conclude that pH present a decisive influence on the SCOA profiles achieved.

CONTENTS

ABBREVIATIONS

AD	Acidification Degree
AD_{Sugars}	Acidification Degree of Sugars
AD_{Total}	Acidification Degree of Substrate
AF	Acidogenic Fermentation
AnD	Anaerobic Digestion
AS	Acclimatized Sludge
ATP	Adenosine Triphosphate
COD	Soluble Chemical Oxygen Demand
COD_{In}	Soluble Chemical Oxygen Demand entering the reactor
cf	Conversion factor
CSTR	Continuous Stirred Tank Reactor
CSTR1	Continuous Stirred Tank Reactor without pH Control
CSTR2	Continuous Stirred Tank Reactor with pH Control
FS	Fresh Sludge
GAP	Glyceraldehyde-3-phosphate
HB	Hydroxybutyrate
HMV	Hydroxymethylvalerate
HPLC	High Performance Liquid Chromatography
HPLC-MS	High Performance Liquid Chromatography coupled to Mass Spectrometer
HRT	Hydraulic Retention Time
HSSL	Hardwood Sulfite Spent Liquor
HV	Hydroxyvalerate
LS	Lignosulphonates
MBBR	Moving Bed Biofilm Reactor
MMC	Mixed Microbial Cultures
OLR	Organic Loading Rate
PHA	Polyhydroxyalkanoates
PHB	Polyhydroxybutyrate

P(HB-*co*-HV)	Poly(hydroxybutyrate-*co*-hydroxyvalerate)
PKP	Phosphoketolase Pathway
PPP	Pentose Phosphate Pathway
PSS	Pseudo-Stationary State
r_p	SCOA Volumetric Production Rate
$-r_s$	Substrate Volumetric Uptake Rate
SCOA	Short-chain Organic Acids
SRT	Sludge Retention Time
SSL	Sulfite Spent Liquor
SSSL	Softwood Sulfite Spent Liquor
TSS	Total Suspended Solids
VSS	Volatile Suspended Solids
WWTP	Wastewater Treatment Plant
X5P	Xylulose-5-phosphate
$Y_{SCOA/S}$	Yield of SCOA on Substrate
$Y_{SCOA/Sugars}$	Yield of SCOA on Sugars

FIGURES

TABLES

1. Introduction

The main driving force for the development of societies along the history was the use of non-renewable resources, such as fossil fuels. Some considerable problems emerged along the years, such the limitation of these resources, due to long recycle times, causing the rise of costs for energy and commodities, and the release of major quantities of carbon dioxide into the atmosphere (S. Bisaria and Kondo, 2014). Since the world economy is heavily dependent on the price of petroleum, its correct management and preservation is crucial to maintain the economic stability, specially taking into account the growth of the world population. The safeguarding and management of world resources are fundamental political tasks to foster a sustainable development in the 21st century (Kamm et al., 2010).

Sustainability is defined as a society capacity to endure through renewal, maintenance or sustenance and concerns not only to environmental but also economic and social issues. Taking into account the dependence of our society on non-renewable resources, and in order to maintain them available and to avoid polluting earth, there has been a demand, in the last years, for new solutions to decrease the consumption of fossil resources. This could be accomplished by developing more sustainable industries focused on the maintenance and management of the available resources worldwide (Kamm and Kamm, 2004; S. Bisaria and Kondo, 2014). The goal is to gradually change the production of commodities and services from fossil to renewable raw materials, which requires new approaches in research and development. Biological, economical, chemical and physical sciences, in addition to process engineering, will play a leading role in the development of the new industries and a strong synergy between these fields is needed. Therefore, the development of new industries, such as biorefineries, based on renewable resources are the key for the access to an integrated system of production of food and feed, chemicals, commodities and fuels in the future (Clark and Deswarte, 2008; Kamm and Kamm, 2004).

The global objective of this work is to produce short-chain organic acids (SCOA) from an industrial byproduct, the hardwood spent sulfite liquor (HSSL), through the development and optimization of a biological acidification process, known as acidogenic fermentation (AF), in an anaerobic reactor by mixed microbial cultures (MMC). Furthermore, the use of an aerobic MMC present as an effective approach to the selection of the acidogenic microorganisms which, in combination with the most appropriate operational parameters and reactor configuration, will allow to achieve a better substrate conversion, and

consequently, a greater acidification degree. In this work, the main objectives are to test different reactor conformations, like CSTR and MBBR, as well as to test one of the most decisive parameters in AF – pH..

Finally, it is important to note that this work will enable to reduce the costs of the well-known AF process by the use of a low cost substrate combined with a MMC and yet, accomplish a high content of SCOA which can be used as substrate in many applications, as is referred further on.

2. State of the Art

2.1. Biorefineries

Natural gas, crude oil or minerals, nowadays used for the most varied applications, come from carbon dioxide fixed by plants through photosynthesis millions of years ago. They are of limited supply and the rate of consumption makes their replacement impossible, being considered, in this way, as non-renewable resources (Clark and Deswarte, 2008). In contrast, resources like solar radiation, water, wind and biomass are considered as renewable resources and if handled correctly, are in no danger of being over-exploited. Whereas the first three resources mentioned are nowadays being used as a renewable source of energy, biomass has the advantage of being used not only to produce energy but also to produce value added chemicals and biomaterials (Clark and Deswarte, 2008; Kamm et al., 2010). Biomass is defined as any organic matter available on a renewable or recurring basis and has a complex composition (Kamm et al., 2010). Although the two most known types of biomass are wood and crops (such as wheat, maize and rice), the biomass derived from waste (food waste, manure, among others industries wastes) proved to be a valuable organic reservoir of raw material and must be used according with its organic composition. The fact that biomass is continuously re-growing/regenerating, by taking up the carbon dioxide from the atmosphere on the process of growing (by photosynthesis) and then returning it at the end of the utilization of its derivatives, creating a closed loop, demonstrate that its use as raw material can be sustainable in many levels (Clark and Deswarte, 2008; Kamm et al., 2010). Recently, the use of food crops as raw materials to produce energy, materials and chemicals has been contested due to their application on the food sector, which is associated with economical competition with that sector, rising also ethical problems. On the contrary, wastes and lignocellulosic materials offer a better alternative as raw materials since they avoid such problems.

Biorefineries are industries whose functioning is analogous to oil-based refineries and in which biomass is economically and ecologically converted to produce goods as chemicals, biomaterials and energy (Liu et al., 2012a). In the last years, energy has presented as the trigger for the development of this area (e.g. the production of bioethanol or biodiesel), however more investigation is being made in order to introduce production processes of other chemicals and biomaterials of interest (Clark and Deswarte, 2008; Kamm et al., 2010).

Biomass processing in biorefineries can be made through biological (e.g. anaerobic digestion, microbial fermentation), chemical (e.g. hydrolysis, oxidation) or thermochemical (pyrolysis, gasification) technologies. These technologies complement each other and their combination in an integrated system present significant advantages in respect to flexibility, specificity and efficiency of the processes. For example, while biological processes present the advantage of high selectivity at low processing temperatures, they usually require elaborate pre-processing stages and long processing times; while thermochemical processes are fast but nonspecific and normally require a high energy input (Kamm et al., 2010; S. Bisaria and Kondo, 2014).

Biorefineries went through three different phases of development: Phase I biorefineries present integrated facilities limited to a single feedstock that is converted into a single major product through a single process (e.g., bioethanol or biodiesel production from corn or oils). These type of biorefineries are already in operation and proven to be economically viable. On the other hand, phase II biorefineries are able to produce many end products (energy, chemicals and materials) from a single feedstock through multiple processes and are also in operation nowadays, however not so extensively as the phase I biorefineries. Lastly, phase III biorefineries are the most advanced, since they use more than one type of feedstock to produce various products, by combining processing technologies as chemical and/or biochemical transformations, extractions and separations. The diversity of the products obtained in phase III biorefineries provides not only a high degree of flexibility to the variations on market demands, but also many options to reach profitability and maximize incomes. Furthermore, the fact that they are able to use multiple feedstock brings advantages because it ensures the feedstock availability and offers the possibility of combining the raw materials in order to make the process more profitable (Clark and Deswarte, 2008; S. Bisaria and Kondo, 2014).

Currently, there are four phase III biorefinery systems under investigation and development: the whole crop biorefinery, the green biorefinery, the two-platform concept biorefinery and the lignocellulosic feedstock biorefinery. Regarding the whole crop biorefinery, it is based on the use of cereals, like wheat or maize, and involves the conversion of the entire plant (straw and grain) into energy, chemicals and materials. On the other hand, green biorefineries use green biomass as feedstock, such as green grass, immature cereals or algae, among others, to produce value added products including energy, chemicals, materials

and food. In the two-platform concept biorefinery, the feedstock is separated into two distinct platforms: the sugar platform, which involves biochemical conversion processes and focus on the fermentation of the sugars extracted from biomass feedstocks; and the syngas platform, based on thermochemical processes, in which biomass is converted into gaseous or liquid intermediate chemicals. Both platforms provide energy, chemicals, materials, potentially food and feed, this way making use of the entire feedstock (Clark and Deswarte, 2008; Kamm et al., 2010). The summary of the biorefinery types is represented in Figure 1.

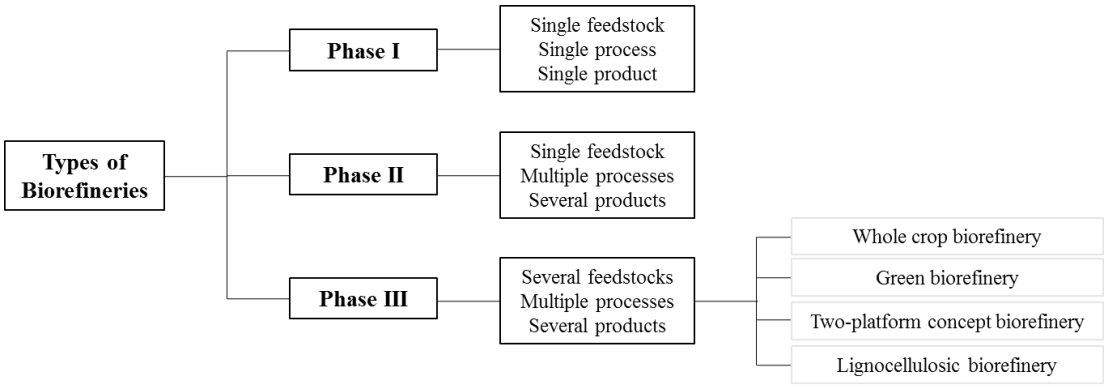

Figure 1. Summary of the existing biorefinery types and characteristics.

Due to the abundance, low cost and broad availability of lignocellulosic biomass, the most promising type of phase III biorefinery is of lignocellulosic feedstock base that can use wood, straw or corn stover to produce chemicals, fuels and energy, among others (Karimi, 2015; Zhang, 2008). In order to develop phase III biorefineries it is necessary to improve, in the first place, the basic biorefinery technologies. The current pulp and paper industries are considered phase I biorefineries and although their sole products are pulp and paper, these facilities are oriented to collect and process large quantities of lignocellulosic biomass, which means that they provide an ideal foundation for the development of advanced phase III lignocellulosic biorefineries (Clark and Deswarte, 2008; Kamm et al., 2010).

Lignocellulosic materials are naturally designed composites that play crucial roles in the survival of plants and consist mainly of polysaccharides, such as cellulose (polymer of glucose; 35-50%) and hemicellulose (heteropolymer of five and six carbon sugars, such as xylose, mannose, arabinose, galactose, ramnose and glucose; 15-35%), and lignin (complex polymer of phenolic compounds; 10-25%), varying the percentages of each component with the plant type (Karimi, 2015; Liu et al., 2012b). The fact that they protect plants against

physical and biological attacks result in a recalcitrant structure and consequently the component fractionation as well as chemical and biological conversions are a challenge to overcome. Cellulose, hemicellulose and lignin interact closely with one another in the cell wall and that results in a unique three dimensional structure, in which hemicelluloses and lignin protect the integrity of the glucose polymer, cellulose (Karimi, 2015; Zhang, 2008). The products that can be possibly obtained from lignocellulosic biomass are represented in Figure 2.

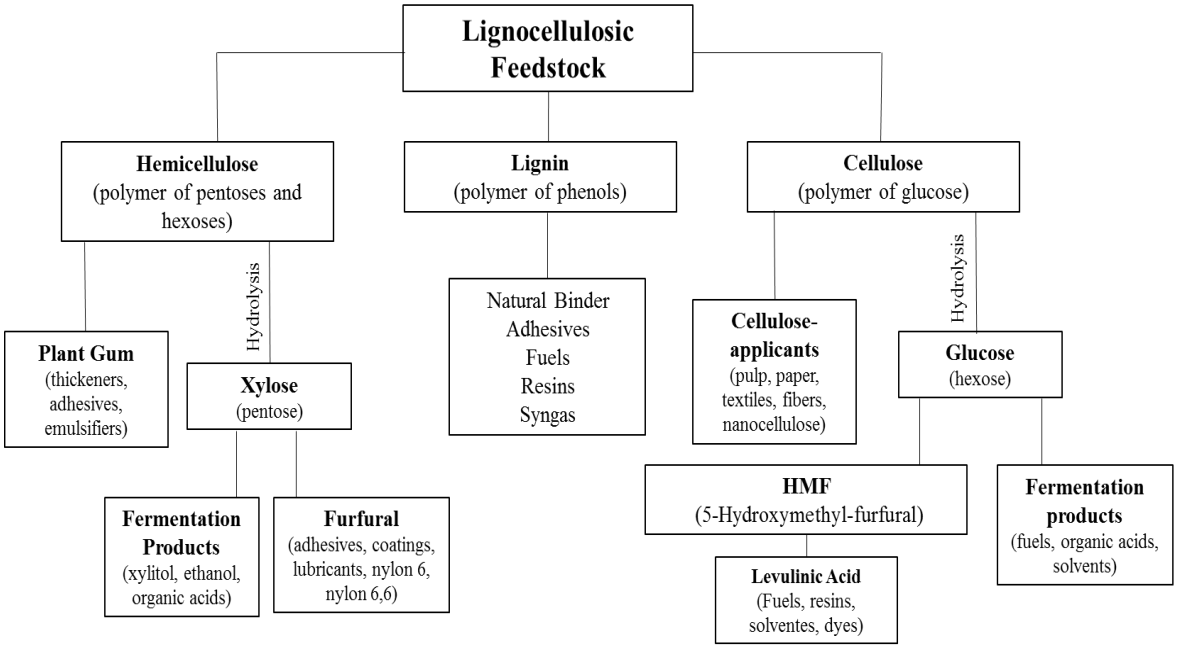

Figure 2. Representation of the products of a lignocellulosic feedstock biorefinery. Adapted from Kamm et al., 2010 and Karimi, 2015.

Basically, cellulose and hemicellulose based products can be categorized into two groups: the first refers to the products that are directly obtained from these biopolymers, such as paper, textiles, cellulose derivatives, packaging films, among others. The second group results from a hydrolysis step (chemical, enzymatic or thermal) that converts the polysaccharides into fermentable sugar mixtures - their building blocks (e.g. five and six carbon sugars), from which can be produced several high-value biobased chemicals or materials (Kamm et al., 2010; Karimi, 2015). In this category, the most known and developed applications of lignocellulosic biomass nowadays are biofuels such as bioethanol, biobutanol or biodiesel, organic acids, polysaccharides and microbial biomass (Karimi, 2015). It is important to note that the production of these chemicals requires far lower quantities of carbon than fuel production, opening this way an economic opportunity for the

development of bio-sourced chemical products through biorefineries, since the value of chemical industry is equivalent to the fuel industry and requires only a fraction of the biomass (S. Bisaria and Kondo, 2014).

In summary, an ideal biorefinery consist on the complete fractionation of lignocellulosic biomass for the production of value-added products, by combining several processes of direct and indirect conversion of these materials.

2.2. Short Chain Organic Acids

SCOA are aliphatic monocarboxylic acids composed by six or fewer carbon atoms (Lee et al., 2014). Due to their low boiling points associated with their low molecular weight, most of these organic acids are considered volatile. The most known and abundant SCOA are acetic, propionic and butyric acids (Zygmunt and Banel, 2009). Also, lactic and valeric acids are considered SCOA. The characteristics of these SCOA are represented in Table 1.

SCOA play a significant role in the metabolism of many living organisms and can be excreted by microorganisms, higher plants and animals (Zygmunt and Banel, 2009). They occur in human colon in which are produced by an anaerobic mixed culture from carbohydrates. Also, SCOA are byproducts of rumen digestion, being absorbed and assimilated as nutrient source by the ruminants, which depend on SCOA for up to 80% of their maintenance energy requirements (Aluwong et al., 2013; Cummings, 1981).

SCOA are mostly used in food and beverages fields as acidifiers but also in the pharmaceutical and chemical fabrication fields. They are commonly used in food industry as taste enhancing additives and preservatives, in the pharmaceutical industry as buffer solutions, in the cosmetics industry in moisturizers, skin-lightening or anti-acne agents and in the chemical industry for the synthesis of biodegradable polymers or as building blocks for the production of many organic compounds such as alcohols, ketones, esters, among others. Also, they have an important role as intermediates in many biological processes (Singhania et al., 2013; Zacharof and Lovitt, 2013). SCOA can be produced by chemical or biological routes either from fossil resources and renewable resources (Yang, 2007). Acetic acid is considered the most important SCOA commercially, since it covers a great part of the market size globally. The major portion of this acetic acid production is made from petrochemical feedstock through chemical processes, such as acetaldehyde or ethylene oxidation or methanol carbonylation (Zacharof and Lovitt, 2013).

Table 1. Representation of the most abundant SCOA characteristics, such as chemical formula, molecular mass, boiling point, pKa, market size, price per tonne and applications. Based on Yang, 2007 and Zacharof and Lovitt, 2013..

SCOA	Chemical Formula	Molecular Mass	Boiling Point (ºC)	pK_a	Market Size (tonnes/year)	Price per tonne ($)	Applications
Acetic	CH_3COOH	60.05	118	4.79	3 500 000	400-800	Biodegradable polymers, adhesives, food additive, solvent, ester production
Propionic	CH_3CH_2COOH	74.08	141	4.87	180 000	1500-1650	Animal and human food additive, chemical intermediate, solvent
Butyric	$CH_3(CH_2)_2COOH$	88.11	163	4.82	30 000	2000-2500	Food additive, pharmaceuticals, animal feed supplement, fishing bait additive
Lactic	$CH_3CHOHCOOH$	90.08	122	3.86	120 000	1000-1800	Cosmetics, food-beverage additive, biodegradable polymers, buffering agents

2.3. Biological Production of SCOA

Like other commodity chemicals, SCOA are nowadays being produced by chemical routes, nevertheless the biological production of these acids is a preferable strategy from the sustainable development point of view due to problems associated with the excessive use of non-renewable resources (Lee et al., 2014; Silva et al., 2013). In the biological production of SCOA, pure sugars such as glucose or sucrose have been commonly employed as the main carbon sources. Though the use of these raw materials increase the economical and ethical problems related to the use of food to produce chemicals. The use of organic-rich wastes (e.g. food waste, wastewaters, wood waste, among others) to produce SCOA provides a sustainable alternative route, reducing, thus, the constantly increasing amount of waste generated (Lee et al., 2014). There were some studies using organic-rich wastes coming from

many industries, and some examples are olive oil mill effluents (Dionisi et al., 2005), food waste (Jiang et al., 2013; Lim et al., 2008; Yin et al., 2016), waste activated sludge (Jankowska et al., 2015; Ma et al., 2016) , pulp and paper mill effluents (Bengtsson et al., 2008), cheese whey (Bengtsson et al., 2008; Gouveia et al., 2016; Silva et al., 2013), sugar cane molasses (Duque et al., 2014) among others (Lee et al., 2014).

The biologic production of SCOA is based on an anaerobic process known as anaerobic digestion (AnD), represented in Figure 3. This process present advantages when compared to aerobic digestion, since it supports high organic loads and has low energy, operation and space requirements (Demirel and Yenigun, 2002). AnD is a sequential biochemical process wherein the complex organic compounds present in the waste, such as polysaccharides, lipids and proteins, are hydrolyzed and fermented into intermediate products that are finally converted into methane and carbon dioxide. This process is therefore composed by four stages, that occur synergistically, in a successive order in which the product of one reaction becomes the substrate for the next reaction. The stages are, by order, hydrolysis, AF, acetogenesis and methanogenesis. All of the stages are executed by four distinct groups of microorganisms that work in a balanced and sensitive symbiotic relationship (Saady, 2013; Singhania et al., 2013). *Clostridium spp*, *Peptococcus anaerobius*, *Bifidobacterium spp*, *Desulphovibrio spp*, *Corynebacterium spp*, *Lactobacillus*, *Actinomyces*, *Staphylococcus* and *Escherichia Coli* are some groups of microorganisms involved in AnD (Visvanathan and Abeynayaka, 2012).

In hydrolysis, complex organic polymers such as polysaccharides, for example, are fragmented into its simpler organic monomers by the enzymes excreted from the hydrolytic or non-hydrolytic and fermentative bacteria (acidogenic bacteria). Usually, hydrolysis is considered the rate-limiting step of AnD given the difficulty of fragmentation of certain substrates. Then, in AF occurs the fermentation of these monomers into SCOA, such as acetic, propionic, lactic, butyric and valeric acids, ethanol, carbon dioxide and hydrogen by the same group of microorganisms. These bacteria are facultative anaerobes (Visvanathan and Abeynayaka, 2012) and those with highest energetical advantage, since they present the lowest time of replication (close to 30 minutes) and the highest growth rates of all the microorganisms involved in the process. Thus, and considering that the substrate is in its monomeric form (hydrolyzed efficiently), the AF stage hardly becomes the limiting stage of

the process (Aquino and Chernicharo, 2005; Visvanathan and Abeynayaka, 2012; Zygmunt and Banel, 2009).

During acetogenesis, the SCOA produced in the previous stage are converted by acetogenic bacteria into acetate, hydrogen and carbon dioxide. In this stage homoacetogenic bacteria with the capacity of convert hydrogen and carbon dioxide into acetate dominate (Saady, 2013). Lastly, methanogenesis is the conversion of the products obtained in the previous steps into methane and carbon dioxide and is performed by methanogenic microorganisms. This microorganisms can be classified as archaea and restrict anaerobes (Ma et al., 2005; Visvanathan and Abeynayaka, 2012), and subdivided into two groups: acetoclastic methanogenic and hydrogenotrophic microorganisms. The first one present low growth kinetics (replication time of two to three days) and an extreme sensitivity to environmental changes, and convert the acetate into methane. The second group also produce methane, although from the conversion of the carbon dioxide. These group present a faster growth (replication time of at least 6 hours) and contribute for nearly 30% of the methane achieved at the end of the process (Aquino and Chernicharo, 2005; Jie et al., 2014).

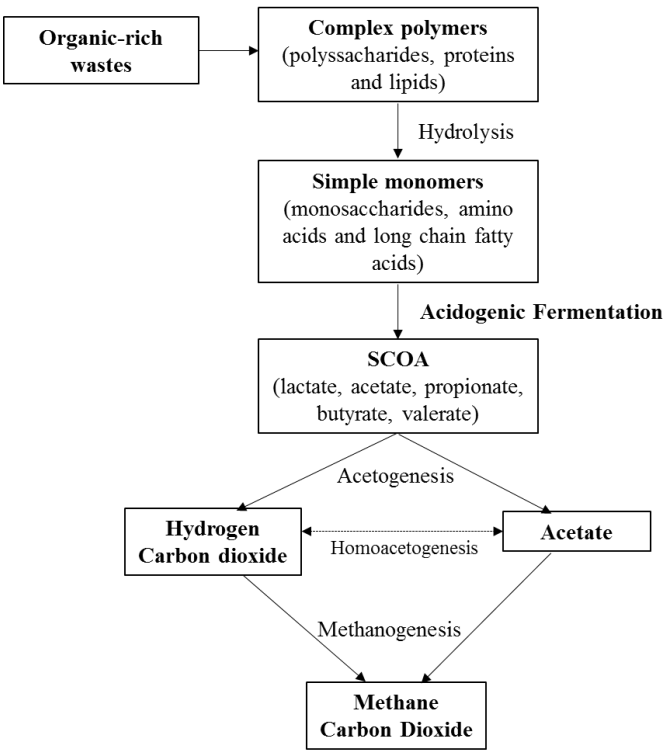

Figure 3. Representation of the AnD process. Based on Lee et al. (2014) and Saady (2013).

In conventional AnD applications, the four stages of the process take place in one single reactor with the acid-forming and methane-forming microorganisms being kept together. In this system, it is crucial to maintain a delicate balance between these two groups of microorganisms since they present significant differences at many levels, such as physiology, growth kinetics, substrate uptake kinetics, nutritional needs and sensitivity to changes in the environmental conditions (Demirel and Yenigun, 2002; Silva et al., 2013). While methanogenic microorganisms need more specific requirements as regards to the nutritional needs and to the conditions of growth and survival, thus being more vulnerable to changes in environmental conditions and inhibition factors, acidogenic bacteria are a more resistant group of microorganisms (Silva et al., 2013). In this way, the co-culture of the acidogenic and methanogenic microorganisms in a single reactor is associated with problems of stability and control already described in the conventional applications of AnD (Demirel and Yenigun, 2002). In order to overcome this problem, Pohland and Ghosh, (1971) first proposed a physical separation of the AnD into two separated reactors connected in series, being the first one destined for AF and the second one for methanogenesis (Pohland and Ghosh, 1971). Thus, the separation of the acidogenic and methanogenic populations was possible, which lead to the achievement of optimal conditions for each group of microorganisms and consequently the increase of the stability and productivity of each stage of the process (Demirel and Yenigun, 2002; Silva et al., 2013).

Even with the separation of AnD process, favoring the yield of AF and consequently, the SCOA production, it is essential to improve the limiting step (hydrolysis), thus enabling an efficient conversion of the organic content present in the substrate. Also, the inhibition of methanogenic bacteria, that could be present in the first steps, is an important factor to improve the production of SCOA, and can be achieved by the control of the operating conditions in AF. Finally, the fact that the AF of rich-organic wastes can produce value-added chemicals, more precisely SCOA, is of major interest since this is not only presented as a new perspective of the use of AnD, that is commonly performed to obtain methane, but also involves the use of byproducts from industries, low cost substrates, dropping the cost of the process (Lee et al., 2014). The acidogenic potential of an organic waste stream, which is the amount of SCOA that can be produced from the fermentation of its organic compounds, and the knowledge of SCOA profiles are critical parameters for the

establishment of local-based biorefinery concepts capable of produce value-added SCOA (Silva et al., 2013).

2.4. Operational Conditions of AF

The process of AF is strongly affected by the operating conditions such as the origin of the inoculum, temperature, pH, nutrients, organic loading rate (OLR), hydraulic retention time (HRT) and sludge retention time (SRT). Their optimization and control are crucial for the success of acidification. Consequently, it is necessary to establish a strategy that combines the use of the appropriate type of reactor with its optimal operational conditions. Furthermore, it is important to refer that the effect of these conditions in AF depends significantly on each other and on the substrate used (Bengtsson et al., 2008; Jankowska et al., 2015).

2.4.1. Origin of the Inoculum

The production of SCOA can be carried out efficiently by MMC. These cultures are microbial populations present in wastewater treatment tanks, with unknown composition which are able to execute specific intra- and extracellular reactions. In order to induce MMC to produce the desired compounds, it is necessary to apply a selective pressure to the biological systems, thus providing some competitive advantage and selecting the microorganisms capable of produce those compounds (Dias et al., 2006). The use of MMC is advantageous when compared to pure cultures since there is no need of a sterile environment, a major operation control and equipment requirements, which reduces significantly the cost of the process. Moreover, pure cultures are not able to convert complex substrates, and that makes the use of low cost substrates impracticable (Tamis et al., 2015). On the contrary, MMC have the ability to convert these substrates efficiently, and can be used with a wide range of low cost substrates (Queirós et al., 2014).

The MMC used in AF can be aerobic or anaerobic (Wang et al., 2014). In general, the cultures used for AF are anaerobic MMC since AnD is an anaerobic process. However, aerobic MMC present great potential for the production of SCOA. The fact that aerobic cultures are present in aerobic tanks which are subjected to extreme conditions (such as climacteric changes) when compared to anaerobic tanks, suggests that aerobic cultures are more robust than anaerobic cultures and thus, its use as inoculum for AF brings advantages to the operational control of the biological system. Furthermore, as referred before,

methanogenic microorganisms are restrict anaerobes and aerobic MMC are composed by aerobes and facultative anaerobes. So, the selection of acidogenic bacteria from these mixed cultures could be possible and become more efficient since it is a way to inhibit the growth of methanogens.

The metabolisms of conversion of glucose and xylose of MMC are of major interest, since these are the main sugars present in various industrial byproducts, such as HSSL, later presented in subchapter 4. When compared to glucose, xylose is not so readily used by the microorganisms, which can be explained by the biochemical pathways of the monomeric sugars. While glucose is used directly in glycolysis (metabolized by the Embden-Meyerhoff-Parnas pathway), Figure 4, xylose needs to be converted first into xylulose-5-phosphate (X5P), which is subsequently metabolized through two possible pathways: pentose phosphate pathway (PPP) or phosphoketolase pathway (PKP), as can be seen in Figure 5. In the PPP, X5P is rearranged and converted into glyceraldehyde-3-phosphate (GAP), which is an intermediate of the glucose metabolism. On the other hand, in the PKP pathway, the phosphoketolase cleaves X5P into GAP and acetyl phosphate, that is further converted into acetate with the generation of one ATP. Generally, the PPP is the most common pathway, nevertheless there are many bacteria with the ability to catalyze the PKP (Temudo et al., 2009).

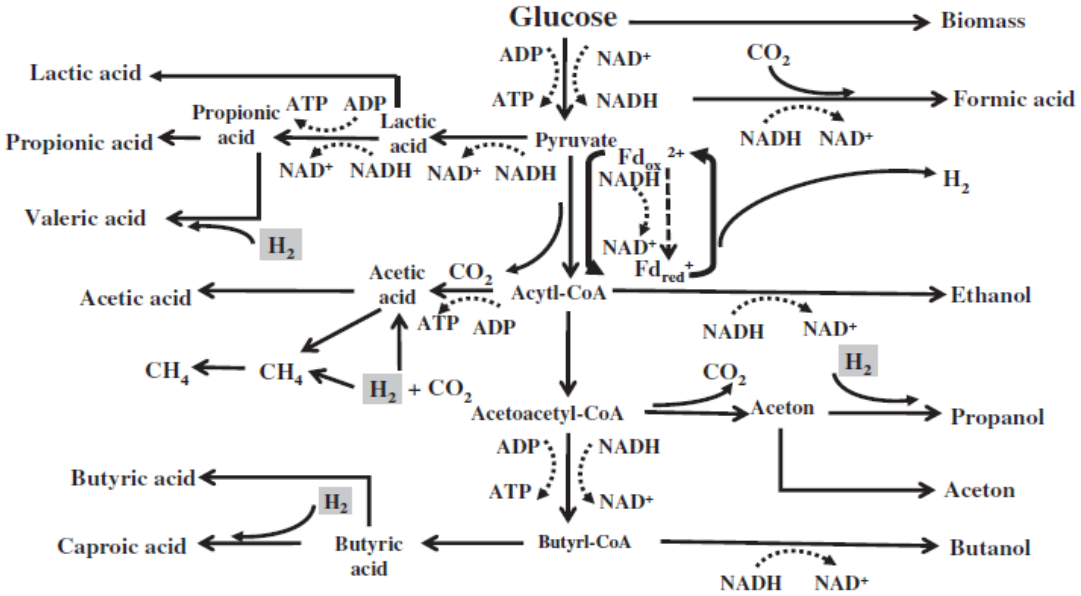

Figure 4. Representation of the metabolism of conversion of glucose by MMC and the products obtained. From Saady, 2013.

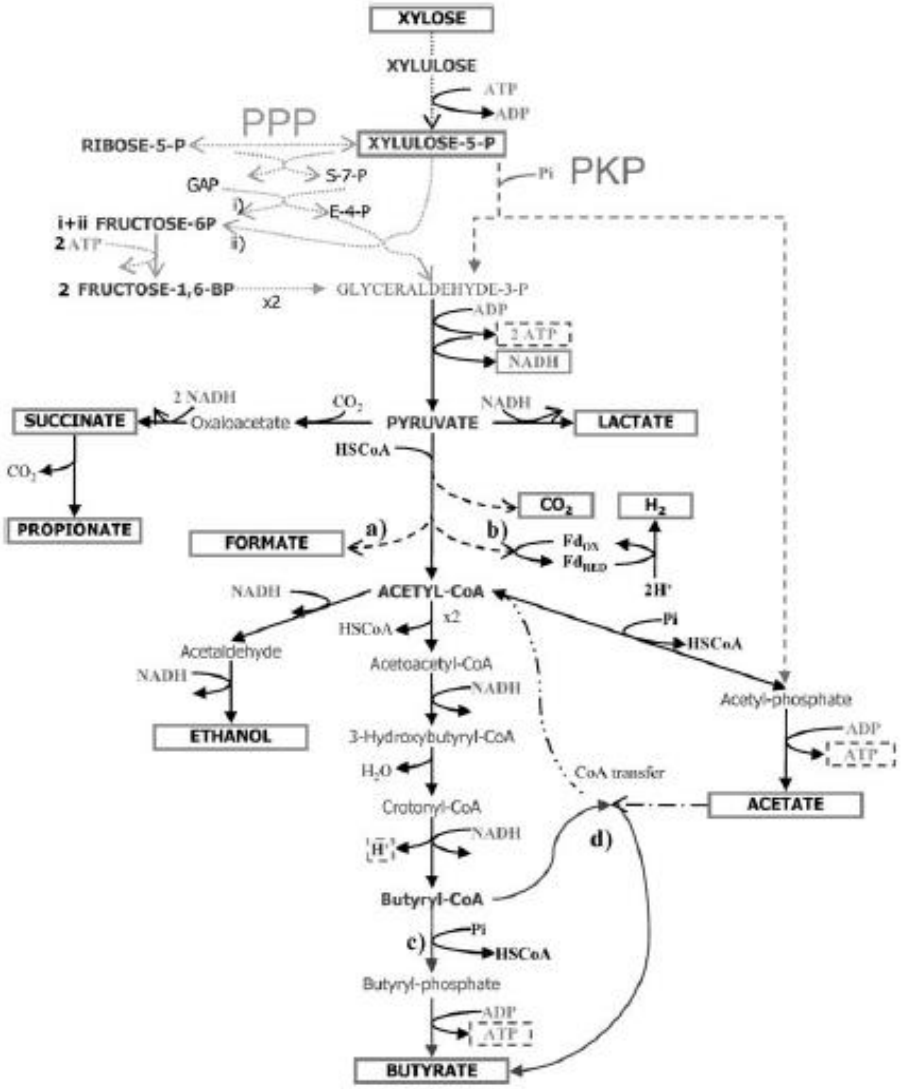

Figure 5. Representation of the metabolism of conversion of xylose by MMC and the products obtained. From Temudo et al., 2009.

2.4.2. Temperature

Temperature is one of the essential parameters to monitor in the AF process and can be subdivided into three ranges: psychrophilic (0-20ºC), mesophilic (20-42ºC) and thermophilic (42-75ºC) (Lee et al., 2014; Rajeshwari et al., 2000). In general, microorganisms involved in AF tolerate well changes in temperature since they do not surpass the upper limit value in which the decay rate start to exceed the growth rate. It is known that for mesophilic range, the bacterial activity and growth declines to half for each 10ºC drop under 35ºC. That means that the lower the temperature, the lower the activity of bacteria and consequently, the longer the conversion time of organic matter (Rajeshwari et al., 2000). On the contrary, the increase of the temperature within the psychrophilic and

mesophilic ranges is beneficial since it increases the concentration of SCOA produced, the rate of SCOA production and the SCOA yield. This results from the fact that the solubility of substrates increases with the increase of the temperature (Feng et al., 2009; Yu and Fang, 2003). Nevertheless, compared to acetogenic and methanogenic bacteria, acidogenic bacteria are resistant to temperature changes which means that this parameter does not affect in great extent the AF stability (Lee et al., 2014). Nevertheless, operation at a thermophilic temperature requires major costs due to the high energy input necessary to maintain the high temperatures throughout the process. Thereby, the most appropriate range for this process is mesophilic, since is a range in which AF is stable and efficient, plus the fact that these temperature range does not require great energy input (Jiang et al., 2013). Relatively to the types of SCOA produced, temperature does not affect them significantly, especially in mesophilic range (Lee et al., 2014).

2.4.3. pH

The values of pH chosen to perform acidification of organic wastes are decisive not only to the success of AF but also to the SCOA profiles obtained in the process. Hence this parameter is known to be a key factor of AF and the study of the optimum pH value is determinant for each substrate used (Tamis et al., 2015).

Although methanogenic bacteria are extremely sensitive to pH variations, having their optimum between 6.8 and 7.2, acidogenic bacteria have a wider range of pH values in which their activity and growth are not affected. However, extreme acidic (pH 3) and alkaline (pH 12) conditions are known to be responsible for inhibit acidogenic bacteria, and thus, these values of pH should be avoided (Jie et al., 2014). Taking into account the separation of AnD into two separate reactors, it became possible to use the optimum pH values for the microorganisms present in each reactor and, consequently, improve their performance (Rajeshwari et al., 2000).

The optimal pH values for AF depend strongly on the substrate used, ranging from 5.25 to 11. For example, Jie et al., (2014) showed that for the production of SCOA from excess sludge the optimum pH value was 10.0. In general, for this type of substrate, an alkaline value of pH in a range of 8-11 is desirable, not only because at these values the activity of methanogens suffers inhibition but also due to the fact that alkaline conditions promote the hydrolysis of sludge, thus increasing the availability of the soluble substrate to conversion (Jie et al., 2014; Lee et al., 2014). On the contrary, Jiang et al., (2013)

demonstrated that a neutral pH value of 6 was the optimum for the production of SCOA from food waste. Also, they showed that extreme acidic values of pH, near to 3, lead to a low SCOA concentration, which can be explained by the fact that at this value of pH the SCOA are undissociated. Thus, microbial growth suffers inhibition since more energy is required for maintaining intracellular pH by actively pumping out undissociated SCOA that diffuse over the cell membrane into the cell (Jiang et al., 2013; Tamis et al., 2015). Relatively to the production of SCOA from cheese whey, more acidic pH values are required to a better performance of conversion, with an optimum between 5.25 and 5.5, while for paper mill effluents, an optimum range of 5.5-6 is required (Bengtsson et al., 2008).

The pH values chosen for the biological production are also determinant for the types of SCOA produced. For instance, for dairy wastewater, the production of propionic acid is enhanced at pH 4-4.5 whereas the production of acetic and butyric acids is favored at pH 6-6.5 (Yu and Fang, 2002). On the other hand, for cheese whey, the opposite happened since the propionic production increased when pH increased from 5.25 to 6 while the acetic and butyric production decreased. In the case of pulp mill effluent, butyrate and propionate increased with pH in the range of 4.9-6, whereas acetate decreased (Bengtsson et al., 2008). Albuquerque et al., (2007) reported that the decrease of the pH value from 7 to 5 in the acidogenic fermentation of molasses lead to the decrease of acetate and propionate concentrations and to an increase in butyrate and valerate concentrations. Liang and Wan, (2015) demonstrated that in the mixed fermentation of brewers spent grain, with pH uncontrolled, the value of pH dropped from 6.5 (the initial value) to 3.8 in one day and was kept in this value for the rest or fermentation. In this case lactic acid was the dominant component through the whole fermentation. It was also demonstrated that a neutral pH value lead to the consumption of lactic acid to produce other SCOA. It is known that lactic acid bacteria are resistant to extremely low pH conditions which suggests that they become dominant in these conditions (Itoh et al., 2012). Furthermore, Temudo et al., (2008) demonstrated that depending on the pH range of operation, different groups of microorganisms become dominant, which has a direct outcome on the types of SCOA produced.

It is important to note that since the work involves the production of SCOA, the accumulation of SCOA such as acetic, lactic, propionic, butyric acids should be avoided since they are responsible for causing the extreme low pH values in the system and therefore,

could inhibit the AF process. For that, in most cases the pH value in the reactor is controlled by the addition of reagents such as bases, in order to maintain a stable value and maximize the SCOA production. Normally, sodium bicarbonate is used to supplement the alkalinity of the medium since it is the only chemical that is able to shift the equilibrium to the desired pH value without disturb significantly the physical and chemical balance of the microbial population (Rajeshwari et al., 2000).

2.4.4. Nutrients

The acidogenic microorganisms involved in AF need micronutrients and trace elements, such as nitrogen, phosphorous, sulphur, potassium, calcium, magnesium, iron, nickel, among others, to keep an optimal growth. These elements are needed in low concentrations, but its lack affects negatively the growth and performance of microbial population. Normally, the nutrient concentration in the feed should be adjusted to a value equal to twice the minimal nutrient concentration needed, thus ensuring an excess of nutrients in the reactor (Rajeshwari et al., 2000).

2.4.5. OLR

The OLR is defined as the amount of organic matter entering the reactor daily per unit of reactor volume, and is usually expressed in terms of soluble chemical oxygen demand (COD). The influence of OLR in SCOA production is not quite understood in literature, but in some studies and depending on the type of substrate, there is an optimum range of OLR considered for SCOA production. In general, the yield of AF increase with the increase of OLR until a point where the OLR value becomes inhibitory and the SCOA concentration drops drastically (Lee et al., 2014). For example, for starchy wastewater, the SCOA concentration increased linearly with OLR with values from 1 gCOD/L·d to 32 gCOD/L·d (Yu, 2001). Furthermore, for chemical synthesis-based pharmaceutical wastewater, the same was observed but with OLR in the range of 7-13 gCOD/L·d. In this case, it was observed that a small increase for 14 gCOD/L·d caused a drastic drop in SCOA concentration (Oktem et al., 2006). For food waste the same tendency was experienced with the increase of OLR from 5 gCOD/L·d to 13 gCOD/L·d, except in this case at the highest OLR value the medium became very viscous which resulted in the instability of the reactor (Lim et al., 2008). Therefore, OLR is a critical parameter in AF since there is an increase in the production of SCOA linearly with OLR until an inhibitory value of the last one. The instability could be

caused by the viscosity of the medium due to the high loading, which affect the rheology and the associated mass transfer implications, resulting in the decrease of SCOA production and in the risk of biomass washout (Jiang et al., 2013; Lee et al., 2014). The achievement of OLR optimum value for operation is crucial to the economic feasibility of the process, since it enables higher rates of conversion.

Jiang et al., (2013) showed that OLR affects the distribution of SCOA obtained, wherein the increase of OLR lead to an increase on acetate and valerate percentage and lower percentages of propionate and butyrate. However this effect is not always observed, since in the case of synthetic dairy wastewater, an increase of OLR of 4 gCOD/L·d to 24 gCOD/L·d lead to an increase in propionate and to the decrease of acetate (Yu and Fang, 2002). In the case of the study involving starchy wastewater already mentioned above, the increase of OLR lead to an increase on butyrate and a decrease on propionate, while acetate remained as the primary SCOA in the both OLR values (Yu, 2001). The inconsistency of these results suggests that although OLR strongly influence the types of SCOA produced, this influence depends not only on the other parameters of operation, but also on the type of waste used and its composition.

2.4.6. Retention Time

In the AF process, the retention time of the substrate and of the culture are operational parameters that affect greatly the success of acidification. The retention time of the substrate in the reactor is defined as HRT, while the one of the culture is defined as SRT.

In general, longer HRT are recommended for AF processes, since they allow for more time for the culture to adapt and convert the substrate efficiently. This is important because most of the substrates used for AF are extremely complex, not easily biodegradable and the culture not always can quickly adapt and convert them. Moreover, shorter HRT can lead to the washout of biomass. One major problem related to this is that operation with higher HRT requires large reactors, thus increasing significantly the cost of the process (Lee et al., 2014). From another point of view, shorter HRT prevent the growth of methanogenic microorganisms, since they have low growth rates compared to acidogens (Jankowska et al., 2015). Consequently, the choice of the most appropriate HRT must take into account these factors in order to achieve a satisfying yield in SCOA production with the lowest cost possible.

Likewise pH and OLR, HRT also affects the type of SCOA produced in AF. Bengtsson et al., (2008) demonstrated that for cheese whey, the increase of HRT from 8h to 95h promoted the production of propionate and suppressed the production of butyrate. For paper mill effluent the tendency was the same, so with the increase of HRT from 11h to 24h the production of propionate was enhanced, whereas the production of butyrate decreased. For both substrates, the production of acetate was not affected by HRT (Bengtsson et al., 2008). Finally, along with pH, this parameter is critical to control the types of SCOA produced, which is extremely important for SCOA applications.

SRT is the time that culture remain inside the reactor. In the case of an operation of a reactor without a biomass recycling system, SRT equals HRT. When a biomass recycling system is used, SRT is longer than HRT.

2.4.7. Reactor Conformation

The choice of the reactor conformation must take into account the requirement of less capital, less area, less necessity of operation and also must be the most reliable and efficient choice when compared to other well established options. Thus, the system must be able to support high OLR and HRT with the minimum operation and maintenance requirements (Rajeshwari et al., 2000). For example, the operation of reactors in batch mode is not recommended, since it implies an accumulation of SCOA and, consequently, an extreme drop on pH to inhibitory values, which can only be suppressed by the constant addition of reagents, and as mentioned before, the system can become economically infeasible.

There are two common technologies used for SCOA production: the suspended growth and attached growth. In suspended growth, the biomass grows freely in suspension. On the other hand, in attached growth there are a support in the reactor in which the biomass attaches, thus preventing the risk of washout. For each technology there are some reactor conformations with different characteristics used nowadays. Continuous stirred tank reactor (CSTR) for suspended growth, and moving bed biofilm reactor (MBBR) for both suspended and attached growth are some examples of the reactors that can be used to perform AF (Lee et al., 2014).

i. CSTR

CSTR is a reactor with suspended growth (no biomass retention) whose mode of operation is continuous, which means that there is a stream with the feed being pumped into the reactor and a stream pumping out the effluent, both with the same flow rate with the volume of the reactor remaining stable through the operation time (Fonseca and Teixeira, 2006). In CSTR systems there is a complete mixing of substrate and biomass, generally obtained mechanically with the help of magnetic stirrers (in the reactors with lower volume), impellers or baffles (Lee et al., 2014). A CSTR system is represented in Figure 6.

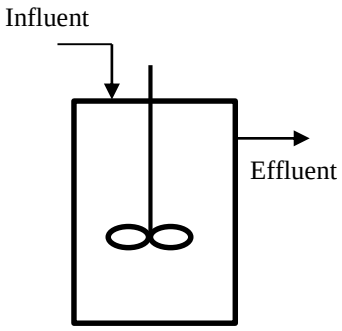

Figure 6. Schematic representation of a CSTR system.

The velocity of agitation should be chosen to guarantee the complete mixing of biomass and substrate without causing damage to the microorganisms by shear stress. The complete mixing in CSTR indicates that the composition of the substrates and products is the same in each point of the reactor and also that the effluent stream will have the same composition that is present in the CSTR (Fonseca and Teixeira, 2006). In addition, in most CSTR systems, the HRT equals the SRT, since the biomass that did not adapt to the substrate and the excess are removed in the effluent. The HRT used must be longer enough for the microorganisms to adapt to the system, thus preventing washout and the failure of the process. Furthermore, there are some cases in which CSTR are coupled with a system of biomass recirculation in order to avoid washout, and in this cases, SRT is higher than HRT (Ozgun et al., 2013).

Normally in CSTR is recommended that after the inoculum the system remains in the batch mode for a few hours or even days, according with the complexity of the substrate, in order to give the culture some time to adapt to the substrate and to the conditions imposed (Fonseca and Teixeira, 2006).

ii. MBBR

The risk of biomass washout that is present in other operational configurations without retaining biomass systems can be avoided by the introduction of new technologies of biomass retention. One way to retain biomass inside the reactor is by using mobile supports in which biomass can attach and grow, forming a biofilm in the surface of these supports (Fonseca and Teixeira, 2006; Karadag et al., 2014). In MBBR, biomass can be effectively retained inside the reactor by the attachment to carrier materials, which are in constant movement and dispersed through the system, thus providing a higher surface for growth of the attached microorganisms and also for the conversion of the usually complex substrates. MBBR incorporates the best characteristics of processes with growth of biomass in suspension and adhered biomass (biofilm) (Karadag et al., 2014; Oliveira et al., 2014).

In MBBR systems, the reactor is filled with carriers and their agitation can be made by a mechanical stirrer or biogas agitation (Figure 7).

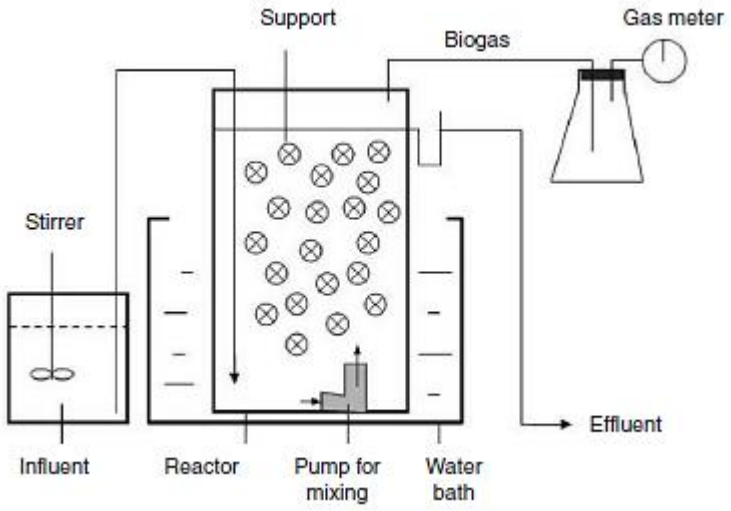

Figure 7. Schematic representation of a MBBR system in a common anaerobic treatment system. From Sheli and Moletta, 2007.

The biofilm carrier element is an important component of the MBBR and the parameters such the density, specific surface area, filling fraction (volume of carrier in empty reactor, usually from 20% to 70%), porosity, durability and material are critical parameters to consider when selecting the appropriate carrier for the desired goal. This because these parameters determine the capability of biomass attachment and the treatment efficiency of MBBR. It is noteworthy that the density of the media with biofilm should be similar to the

density of water, thus spending less energy for agitation (Oliveira et al., 2014; Sheli et al., 2014; Sheli and Moletta, 2007). The carriers used for the attachment of the microorganisms can be composed by different materials. Polyethylene and polypropylene cylindrical rings, polyurethane foam, polyurethane-activated carbon, polyvinyl-alcohol gel, among others, are examples of the diversity of carriers that can be used in the MBBR systems for the attachment of the biomass (Sheli et al., 2014).

Biofilm reactors present advantages such as high loading capacity, concentration of biomass and resistance to hydraulic or organic overloads. Compared to another systems, MBBR are presented as a better choice since they have significantly reduced start-up times and increased organic loading rates, thus being more stable and incorporating the advantages of biofilm technology in a compact reactor (Karadag et al., 2014; Sheli and Moletta, 2007). The fact that in MBBR the system can be quickly restored in the case of a shock load is also of great interest (Rajeshwari et al., 2000). In addition to that, the longest SRT maximizes the conversion rates and reduces the required reactor volumes, which economically present as a great advantage, since it is possible to operate with smaller reactors and yet, have the same yield when compared to the other systems without biomass retention (Oliveira et al., 2014).

2.5. Applications of Biologically Produced SCOA

The SCOA produced from AF of rich-organic wastes are a valuable substrate which can be used by microorganisms as carbon source. Therefore, they have an extreme importance as precursors in many applications, such as for the production of bioenergy (e.g. hydrogen and methane), for biological nutrient removal (e.g. denitrification) and for the production of biodegradable bioplastics such as polyhydroxyalkanoates (PHA) (Silva et al., 2013). It is important to refer that the types of SCOA obtained in the effluent of AF are of main interest for these applications, since different SCOA can provide different conversion efficiencies and in some cases, different final products, which economically and commercially is important. So, according with the application in which the effluent will be used, the concern is not only to obtain high amounts of SCOA, but also to direct the AF process to the production of the most efficient SCOA for each application.

2.5.1. Bioenergy

As described above, SCOA can be used to produce bioenergy through AnD, such as hydrogen and methane. The production of hydrogen via AnD is known as dark fermentation, and in this process the methanogenesis is suppressed to produce hydrogen instead of methane (Arudchelvam et al., 2010). In dark fermentation, acetate and butyrate are the most efficient SCOA for hydrogen production, whereas propionate have an adverse effect on the yield of the process (Gioannis et al., 2013). Moreover, the production of hydrogen through dark fermentation also produces SCOA since this process is based on the first steps of AnD. Thus, there are other processes that could be coupled with this one in order to allow further energy extraction and therefore enhance hydrogen production efficiency. Photofermentation is an example of a downstream process usually coupled with dark fermentation, in which purple non-sulfur bacteria convert SCOA into hydrogen in the presence of light (Chen et al., 2008; Su et al., 2009). It is important to refer that the types of SCOA used have an impact on hydrogen production, since *Rhodobacter sphaeroides*, which is a bacterial strain normally used in photofermentation, metabolize better acetate and propionate when compared to butyrate (Uyar et al., 2009).

As regards to methane production, the type of SCOA used as substrate also affects the efficiency of fermentation. Wang et al., (2009b) demonstrated that for 2.4 g/L of acetate and butyrate, no significant inhibition of methanogenic bacteria activity was observed. On the contrary, for less concentrations of propionate, 0.9 g/L, methanogenic bacteria suffered significant inhibition, resulting in the decrease of bacterial concentration and in the loss of activity.

2.5.2. Denitrification

The use of waste-derived SCOA have presented as an excellent and cost-effective carbon source for denitrification. SCOA readily pass through the cytoplasmic membrane of heterotrophic organisms in the sewage flora to be metabolized internally as a carbon or energy source (Min et al., 2002). Although carbon external sources (e.g. methanol and synthetic acetate) are normally used for denitrification, internal carbon sources present advantages since, for example, the SCOA used can be produced from low cost substrates, making the process more economically feasible. Furthermore, methanol produces less energy and have a lower denitrification rate than SCOA. Also, the use of synthetic acetate

can become expensive, plus the fact that presents lower denitrification rates than waste-derived SCOA (Lee et al., 2014; Singhania et al., 2013). The different biologically produced SCOA show different efficiencies in denitrification process. Denitrifying bacteria have preference for lower molecular weight SCOA, reason why acetate is normally the first SCOA to be consumed (Lee et al., 2014). Thus, the denitrification rate of acetate is two times higher than propionate, which shows that acetate is the most effective SCOA in denitrification (Jiang et al., 2013).

2.5.3. PHA

PHA are biodegradable polymers synthetized by bacteria as intracellular storage reserves of carbon and energy (Lemos et al., 2006; Queirós et al., 2015b). They can be produced using SCOA as source of carbon and present as an alternative to the common plastics due to their similar characteristics plus the fact that they are biodegradable and biocompatible (Lee et al., 2014; Queirós et al., 2014).

The common plastics accumulation in the environment has caused serious concerns around the world, since the degradation of these materials occurs at very low rates. Biopolymers are a feasible alternative to common plastics due to the fact that their production is made from renewable resources, thus adjusting to the sustainability concept (Queirós et al., 2014).

Currently the PHA commercialized are produced by pure cultures, which have the ability to store PHA up to 90% of their cell dry weight. The substrate price, PHA yield and extraction efficiency of the polymer from the cells are critical parameters that are directly correlated with PHA cost, which is still moderately high when compared with synthetic plastics. The difference of cost is the major barrier to the substitution of the conventional plastics (e.g. polypropylene €0.74/kg) for PHA (€2.20-5.0/kg) (Gholami et al., 2016; Koutinas et al., 2014). Having this in mind, there has been an urgent need to reduce PHA production costs over the past few years, and that can be achieved by the combination of the use of low cost substrates, such as industrial wastes, with the use of MMC, which do not require sterile conditions and additional equipment (reducing the necessity of process control), thus reducing the overall costs of the process (Lee et al., 2014; Queirós et al., 2015a). Furthermore, the optimization of the process parameters is crucial to enhance the potential of the low cost substrates, usually quite complex, and to increase the percentage of accumulation of MMC which is lower than with pure cultures.

The production of PHA by MMC is usually composed by three stages: AF, MMC selection and PHA accumulation. In AF, the precursors to PHA synthesis, SCOA, are produced through an anaerobic process. Then, the microorganisms with the capacity to accumulate PHA are selected by applying selective pressure to the reactor, usually by the use of alternate feast and famine regime, in which only the microorganisms that accumulated PHA during the feast phase are able to survive the famine phase, since they can use the stored PHA as carbon source. Lastly, the PHA storage capacity of the selected microorganisms is maximized through the feeding of SCOA in the accumulation stage (Albuquerque et al., 2010; Duque et al., 2014).

In PHA production, the composition of the fermentation products, SCOA, is a key characteristic since it influences the type of biopolymer produced. Through the supply of different compositions of SCOA as substrate for the microbial production of PHA, different polymers are produced, differing from each other in the monomeric composition. By controlling the composition of SCOA used as substrate, it is possible to obtain biopolymers with a broad range of physical properties, which is of major interest to industry and thus can increase the commercial value of the product (Lee et al., 2014; Silva et al., 2013). For example, acetate and butyrate are preferentially stored as a homopolymer of hydroxybutyrate (HB), poly-hydroxybutyrate (PHB), which is highly rigid and fragile. On the contrary, propionate and valerate promote the synthesis of hydroxyvalerate (HV) monomers. By incorporating different monomeric units in the polymer chain, co-polymers with enhanced mechanical properties can be produced (Lemos et al., 2006). Increasing propionate production in AF brings advantages since this SCOA promotes the production of HV monomers, and consequently, the production of co-polymers with HB and HV monomers, poly(hydroxybutyrate-co-hydroxyvalerate), P(HB-*co*-HV). This is of great interest commercially since the co-polymer P(HB-*co*-HV) has characteristics of interest, such as lower melting temperature and higher decomposition temperature when compared to PHB, thus being more flexible. So, the higher the HV content of the co-polymer, the higher the malleability/elasticity and resistance of it (Bengtsson et al., 2008; Shen et al., 2014).

Regarding the efficiency of each SCOA for the production of PHA, Lemos et al., (2006) demonstrated that a higher polymer yield was obtained for acetic, followed by butyric, propionic and valeric acids. The lowest polymer yield was achieved for propionic and valeric acids due to the decarboxylation they require to produce acetyl coenzyme A. In

addition to that, a feeding stream rich in SCOA such as acetic or butyric acids mostly resulted in the formation of the homopolymer PHB. On the other hand, the predominance of propionic or valeric acids, led to the formation of a co-polymer of HV and hydroxybutyrate HB and a terpolymer of HV, HB and hydroxymethylvalerate (HMV) (Lemos et al., 2006).

2.6. Hardwood Spent Sulfite Liquor

In pulp and paper industries, lignin is removed from the wood through pulping processes, in which the pulp used for the papermaking or as a chemical feedstock is produced. There are several processes for chemical pulping, which can occur under strong basic or acids conditions. For example, while Kraft process occurs through alkaline conditions (pH 13-14), the sulfite pulping occurs through acidic conditions (pH 1-2) (Pereira et al., 2013).

Sulfite spent liquor (SSL) is a side product from the acidic sulfite wood pulping and is generally burned for chemicals and energy recovering, after sequentially evaporation to concentrate it. The wood origin determines the chemical composition of SSL, from which its practical applications depend (Marques et al., 2009; Xavier et al., 2010). Various wood species like hardwood, softwood or a mixture of both can be used by the pulp and paper industry. Softwoods (gymnosperms) and hardwoods (angiosperms) differ from each other mainly on fiber morphology and chemical composition. For example, whereas the SSL obtained by softwoods, the softwood sulfite spent liquor (SSSL), contain a high proportion of hexoses (>70%), those achieved by hardwoods, HSSL, is composed mainly by pentoses (>70%). Although the fibers from both types of wood are mainly composed by cellulose, the polymer physical characteristics are different, resulting in liquors completely diverse in composition (Pereira et al., 2013).

The acidic sulfite pulping of *Eucalyptus globulus* wood allows for the production of the bleached pulps for the paper manufacturing, which represent a strong contribution to the economic profits of South Africa, Portugal and Spain (Marques et al., 2009). The byproduct obtained in this process, HSSL, is composed by lignocellulosic materials which are of easy access and low cost due to their large scale production in pulp and paper industry. Furthermore, the main objective of the wood pulping process is the removal of lignin, thus keeping the cellulose and hemicellulose integrity. The extreme conditions, such as high temperatures and acidic pH values in which the acidic wood pulping occurs, causes the

partial hydrolysis of hemicelluloses (Xavier et al., 2010). This way, monomeric sugars are released, making HSSL a potential suitable substrate in many bioprocesses. The fact that HSSL contains a high chemical oxygen demand (higher than 200 gCOD/L) means that it cannot be discharged into natural basins due to the environmental concerns and thus, this liquor must be treated before its disposal (Pereira et al., 2013). In fact, HSSL has already been the target on many studies to the production of second generation bioethanol, in order to substitute the starch-based platforms (first generation bioethanol) (Limayem and Ricke, 2012; Pereira et al., 2013). During the acidic sulfite pulping, which occurs at high temperature (145 ºC) and at a low pH value (pH 1), lignin is sulphonated due to the reagents used and conditions applied. Then, the sulphonated lignin is removed from wood as LS salts (Pereira et al., 2013), which represent most of the composition of HSSL. After the pulping process, the HSSL obtained containing LS and degraded carbohydrates is concentrated by evaporation in a set of 7 evaporators. The composition of HSSL is presented in Table 2. It is important to refer that the data related with the conditions of the sulfite pulping and the composition of HSSL were provided by Caima – Cellulose Industry S.A.

Table 2. Composition and concentration of the HSSL components, in g/L.

Components	Concentration (g/L)
LS	117.7
Acetic acid	15.9
Extractives	10.7
Methanol	1.2
Furfural	0.7
Formic acid	0.8
Ash	19.3
Xylose	38.7
Glucose	7.3
Ramnose	1.1
Arabinose	1.1
Manose	2.1
Galactose	2.1

As can be seen, LS are the major components in HSSL, followed by xylose, acetic acid, extractives and glucose. Also other monomeric sugars are present, but in smaller amounts. The inorganic salts, determined as ash, present a concentration of 19.3 g/L. The

major amounts of xylose and acetic acid results of the extensive degradation of acetylated glucuronoxylan that is the most predominant hemicellulose in hardwoods (Pereira et al., 2013; Xavier et al., 2010).

The composition and broad availability of HSSL makes it a potential candidate to be used as feedstock in many biological processes, which is the case of the AF process.

3. Material and Methods

3.1. Inocula

Two different aerobic MMC, fresh and acclimatized, were used as inoculum. They were collected from an aerobic tank of the wastewater treatment plant (WWTP) Aveiro Sul, SIMRia and from an acidogenic reactor, respectively. The acclimatized MMC was already enriched in acidogenic microorganisms (Queirós et al., Submitted). The biomass concentration of the MMCs were determined by analysis of the total and volatile suspended solids (TSS and VSS), according to Standard Methods (Clesceri et al., 1998).

3.2. Substrate

The HSSL from magnesium based acidic sulfite pulping of *Eucalyptus globulus* was provided by Caima – Indústria de Celulose S.A. (Constância, Portugal). The pre-evaporated HSSL was collected from an inlet evaporator of a set of multiple-effect evaporators to avoid the presence of free SO_2. Before HSSL utilization part of its most recalcitrant compounds were removed using a preliminary pretreatment (Pereira et al., 2012) which started with a pH adjustment to 7.0 with 6 M KOH, followed by aeration with compressed air (6 hours per liter of HSSL). Then, HSSL was centrifuged for 1 h at 5000 rpm and the precipitated colloids were filtered off using a 1.0 µm pore size (VWR 692). The total COD of the pretreated HSSL was determined (229 gCOD/L), being the LS the main components (162 g/L) along with xylose, acetic acid and glucose (33.0, 12.5 and 4.49 gCOD/L, respectively). Finally, the pretreated HSSL was stored at 4 ºC.

3.3. Fermentation Medium

The fermentation medium used was composed by nutrients and the pretreated HSSL. In order to achieve an OLR of 7.62 g COD/L·d (HRT = 2.34 days) firstly and then of 5.95 g COD/L·d (HRT = 3.01 days) in the reactor, HSSL was diluted with a mineral solution (1:12.8). The adjustment of HRT from 2.34 to 3.01 days was carried out only in the CSTR without pH control (CSTR1). Thus, both the CSTR with pH control (CSTR2) and MBBR were operated at a HRT of 3.01 days and with an OLR of 5.95 g COD/L·d. The fermentation medium was composed by, per liter of distilled water: 80 mg of $CaSO_4 \cdot 2H_2O$, 160 mg of $FeSO_4 \cdot 7H_2O$, 160 mg of $MgSO_4 \cdot 7H_2O$, 80 mg of $Na_2MoO_4 \cdot 2H_2O$, 160 mg of NH_4Cl. A

solution with 160 mg/L of KH_2PO_4 and 80 mg/L K_2HPO_4 was prepared separately to avoid precipitation with the magnesium salts during sterilization. The pH of the medium was adjusted to 6.0 with 6 M KOH and the two solutions were autoclaved for 20 min at 121 ºC. Phosphates were added to the feed under sterile conditions and at room temperature. The COD of the fermentation medium was 17.8 gCOD/L.

3.4. Experimental Setup

3.4.1. CSTRs

A CSTR conformation was chosen to accomplish the AF of the pretreated HSSL under anaerobic conditions, as can be seen in Figure 8. Two independent CSTR were operated, one without pH control, CSTR1, and the other with pH control, CSTR2, at 6.0 ± 0.1, 7.0 ± 0.1 and 8.0 ± 0.1, by the addiction of 2 M NaOH and 1 M HCl.

Figure 8. Experimental setup representation of the CSTR systems for the acidogenic fermentation of HSSL. CSTR2 (left) and CSTR1 (right).

The working volume of both CSTRs was 2 L and the flow rate of the influent and effluent solutions was 0.85 L/d for HRT of 2.34 days and 0.66 L/d for HRT of 3.01 days. The flow rate was imposed by an Ismatec^{TM} compact digital multichannel pump. Since the CSTRs had no system for retaining the biomass, the SRT was the same as the HRT. Reactor stirring was performed by a magnetic stirrer and kept constant at 100 rpm. Furthermore,

nitrogen was sparged regularly to assure anaerobic conditions. Both CSTRs worked with temperature control at 30.5 ± 1.0 ºC. The effluent was collected at the outlet of the reactor by overflow. Lastly, the initial sludge concentration in the CSTR1 was 2.65 g/L. For the CSTR2, the initial WWTP sludge concentration was 11.6 g/L.

3.4.2. Effect of initial pH

Batch experiments took place in encapsulated mini flasks with 100 mL of working volume (Figure 9) to study the effect of initial pH value. Two sets of experiments were carried out, one using the acclimatized sludge (AS) from the CSTR1 as inoculum, containing the selected acidogenic population, and another using WWTP fresh sludge (FS). For each set of experiments, six different pH values were tested (pH 4, 5, 6, 7, 8, 9) using appropriated buffer solutions. The initial COD of the flasks for both sets of experiments was 16.7 g COD/L with the fermentation medium described in section 3.3. For the experiment with the AS, the initial biomass concentration was 1.65 g/L, while for the experiment with the FS, the initial biomass concentration was 11.9 g/L. After the inoculation and encapsulation, the medium was sparged with nitrogen to ensure anaerobic conditions. During all the fermentation time, the flasks were maintained at 30ºC and with constant magnetic stirring.

Figure 9. pH batch experiments setup at the end of the fermentation.

3.4.3. MBBR

The MBBR used is showed in Figure 10. The MBBR with a working volume of 3.22 L and a flow rate of the influent and effluent solutions of 1.08 L/d worked with HRT of 3.01 days. The flow rate was imposed by an Ismatec^{TM} compact digital multichannel pump. The reactor was filled with 41 % (1.5 L) cylindrical polyethylene carriers Bioflow 9, supplied by RVT Process Equipment GmbH. Bioflow 9 carriers present a packing density of 145 kg/m^3,

a specific surface area of 800 m^2/m^3 and a dimension of 9x7 mm (diameter x height). The mixing of the MBBR was constant and carried out by a submerged pump (Syncra Silent 1.0, 230V-50Hz, flowrate of 950 L/h) fixed on the bottom of the reactor. The effluent was collected at the outlet of the reactor by overflow. Furthermore, nitrogen was sparged regularly to assure anaerobic conditions. The jacketed MBBR system worked with temperature control at 30.5 ± 1.0 ºC. Lastly, the initial sludge concentration in the reactor was 11.6 g/L.

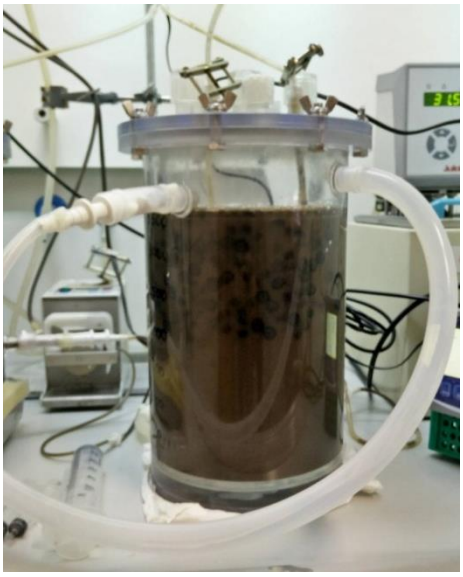

Figure 10. Experimental setup representation of the MBBR system for the acidogenic fermentation of HSSL.

3.5. Sampling

Samples of 5 mL were collected every day from the reactors, two times a day. Then, samples were centrifuged at 13000 rpm for 15 minutes (Centrifuge MiniSpin, Eppendorf) and the pellet was discarded. The supernatant was stored at -16 °C for further determination of glucose, xylose, SCOA, COD and LS concentrations. Additionally, 5 mL samples were collected every day for TSS and VSS determination.

In the batch experiments 1 mL of sample was collected from each flask every day during the first two weeks, and with two days difference until the end of the experiments. The samples were centrifuged and the supernatant was stored at – 16 ºC for further determination of glucose, xylose and SCOA concentrations.

3.6. Analytical Methods

3.6.1. COD

COD was measured accordingly to Standard Methods (Clesceri et al., 1998). The dilution of samples must take into consideration the detection range of the method (100 – 900 mg/L) and replicates were prepared for each sample. In the preparation of blank, 2.0 mL of distilled water was added to the test tubes instead of sample. Then, tubes suffered a vigorous agitation and were placed on a pre-warmed incubator (Spectroquant TR620, Merck Millipore) for 2h at 150 ºC. After the digestion, tubes were taken from the incubator and placed in the dark to cool down to room temperature. Lastly, the absorbance of the tubes was read at 600 nm with a colorimeter (Spectroquant Picco COD/CSB, Merck Millipore). The COD concentrations were then calculated based on a calibration curve performed with glucose standards.

3.6.2. Determination of Sugars and SCOA

High performance liquid chromatography (HPLC) was used to determine the concentration of xylose, glucose and SCOA in the collected samples. 700 µL of sample were filtered with cellulose acetate membrane filters with 0.2 µm pore size (CoStar Spin-x) by centrifugation for 20 minutes at 8000 rpm. Then, 20 µL of sample were injected (Auto-sampler Hitachi L-2200) in an anion exchange column (Rezex^{TM} ROA – Organic Acid H^+ (8%), Phenomenex, 300 x 7.8 mm) connected to a refraction index detector (Hitachi RI L-2490). The column was at 65 ºC in a Gecko 2000 external oven and the eluent used was 0.005 N H_2SO_4, prepared with Milli-Q water, at a flowrate of 0.5 mL/min (Hitachi L-2130).

Along with samples, standard solutions with known concentrations of xylose, glucose and SCOA were also injected to obtain calibration curves. The minimum and maximum concentrations of the standards were 0.15 g/L and 3.0 g/L for lactic, iso-butyric and valeric acids, 0.20 g/L and 4.0 g/L for propionic and butyric acids and 0.25 g/L and 5.0 g/L for xylose, glucose and acetic acids, respectively.

3.6.3. Lignosulphonates

The determination of the content in LS of the samples was carried out in accordance with Restolho et al., (2009). The samples were diluted 1:400 and their absorbance was measured in a UV spectrophotometer (Shimadzu UVmini-1240) at 275 nm. The LS

concentration was calculated based on the Beer-Lambert law, with a $\varepsilon = 7.62\ g^{-1}cm^{-1}$. The samples dilution took into account the linearity zone of the method, in order to obtain an absorbance between 0.1 and 0.7.

3.6.4. Biomass

Biomass concentration was determined using TSS and VSS procedure described in *Standard Methods* (Clesceri et al., 1998). In this procedure, microfiber filters with 1.0 µm pore size (VWR 692) were calcined for 30 minutes at 550 ºC to remove all the organic matter. After cooling down to room temperature, filters were weighted and 5.0 mL samples were filtered using a vacuum pump. Then, filters with the biomass were dried in the oven for 24h at 105 ºC to remove the water and once again weighted at room temperature to achieve the TSS concentrations. Finally, the filters were calcined at the same conditions as earlier, and then weighted at room temperature to achieve the VSS concentrations.

3.7. Calculations

3.7.1. HRT

The HRT was calculated by the division of the reactor working volume for the flowrate of the pump (Equation 1). The SRT was the same as the HRT for both CSTRs. For the MBBR, the SRT was not evaluated.

$$HRT\ (days) = \frac{Working\ volume\ of\ reactor\ (L)}{Pump\ flowrate\ (L/d)} \quad \text{(Equation 1)}$$

3.7.2. OLR

The OLR of the reactors was achieved by the equation presented (Equation 2).

$$OLR\ (gCOD/L \cdot d) = \frac{COD_{In}}{HRT} \quad \text{(Equation 2)}$$

3.7.3. Conversion of Units

The values of SCOAs, xylose, glucose and biomass in g/L were converted in gCOD/L using conversion factors that represent the mass (g) of oxygen required to oxidize 1 g of

compound based on the oxidation reactions for each compound. The overall oxidation equation is represented below (Equation 3).

$$\text{a compound} + \text{b}\ O_2 \rightarrow c\ CO_2 + d\ H_2O + e\ NH_3 \qquad \text{(Equation 3)}$$

In which *a*, *b*, *c*, *d* and *e* represent the stoichiometric coefficients of the equation. Therefore, the conversion factor (cf) was calculated according with the following equation (Equation 4).

$$cf\ (gO_2/g) = \frac{b \times M(O_2)}{a \times M(compound)} \qquad \text{(Equation 4)}$$

The conversion factors were 1.07 g O_2/g for glucose, xylose, lactic and acetic acids, 1.51 g O_2/g for propionic acid, 1.82 g O_2/g for butyric acid, and 2.04 g O_2/g for valeric acid. For biomass it was assumed an empirical molecular formula of $C_5H_7NO_2$ that corresponded to a conversion factor of 1.42 g O_2/g for biomass (Queirós et al., 2014).

3.7.4. Acidification Degrees

The total acidification degree (AD_{Total}) represents the amount of substrate consumed to produce SCOA taking into account all the organic matter entering the reactor (Equation 5). The sugars acidification degree (AD_{Sugars}) represents the amount of sugars consumed to produce SCOA taking into account the xylose and glucose fed to the reactor (Equation 6). These calculations were performed as percentages.

$$AD_{Total}\ (gCOD/gCOD) = \frac{[SCOAs]}{COD_{in}} \times 100 \qquad \text{(Equation 5)}$$

$$AD_{Sugars}\ (gCOD/gCOD) = \frac{[SCOAs]}{COD_{sugars}} \times 100 \qquad \text{(Equation 6)}$$

3.7.5. Yields and Rates

For the effluent, the yield on SCOA was calculated relatively to the COD of the feed, represented by $Y_{SCOA/S}$ (Equation 7) and relatively to consumed sugars (xylose and glucose), $Y_{SCOA/Sugars}$ (Equation 8).

$$Y_{SCOA/S}\ (gCOD/gCOD) = \frac{[SCOA]_{produced}}{(COD_{In} - [SCOA]_{in}) - (COD_{out} - [SCOA]_{out})}$$ (Equation 7)

$$Y_{SCOA/Sugars}\ (gCOD/gCOD) = \frac{[SCOA]_{produced}}{COD_{Sugars\ in} - COD_{Sugars\ out}}$$ (Equation 8)

The substrate volumetric uptake rate ($-r_s$) and the SCOA production volumetric rate (r_p) in g COD/L h were also calculated by dividing the substrate consumed or product formed, respectively, by the HRT.

4. Results and discussion

4.1. Selection of inoculum

Since AF is a stage of AnD, traditionally, an anaerobic MMC is chosen to perform the process. However, in this case, and taking into account that one of the major problems associated with the AF is the growth of methanogens, an aerobic MMC was chosen as inoculum. In this way, it was possible to avoid a possible growth of methanogenic microorganisms (Gerardi, 2003), which use the SCOA as substrate to produce methane and carbon dioxide and, consequently, affect negatively the yield of the process. Since methanogens are strict anaerobes, their existence in an aerobic population is unlikely (Visvanathan and Abeynayaka, 2012).

For these reasons, an aerobic MMC was chosen to be used in this study to select efficiently the acidogenic populations minimizing the risk of growth of methanogens. The initial biomass concentration used to inoculate the bioreactors and flasks was the maximum as possible. Thus, only facultative and acidogenic microorganisms would be able to survive, with the strict aerobes being washed out from the system due to the anaerobic conditions imposed. Such strategy is associated with major losses of biomass in the beginning of the AF (Fernández-Morales et al., 2010; Queirós et al., Submitted).

4.2. CSTR1

4.2.1. Choice of the operational conditions

CSTR1 was used to perform AF without pH control. The inoculum used in the CSTR1 had the concentration of 2.65 g/L and was already rich in acidogenic microorganisms since it came from an acidogenic reactor (Queirós et al., Submitted). Thus, the conditions chosen to apply to the CSTR1 were based on the conditions imposed to the reactor that originated the inoculum. This acidogenic reactor was fed with the same carbon source used in the current study and worked at an HRT of 1.76 days, OLR of 11.8 gCOD/L·d and at a mesophilic temperature of 30ºC (Queirós et al., Submitted).

The COD concentration of HSSL was 229 g/L. The feed presented a COD concentration of 17.8 g/L, being 0.35 gCOD/L glucose, 2.58 gCOD/L xylose and 0.97 gCOD/L acetic acid. The OLR applied was 7.62 gCOD/L·d at an HRT of 2.34 days. Temperature was kept in the mesophilic range at 30 ºC allowing the process to occur without

major energetic needs and still with good efficiencies (Gruhn et al., 2016; Lee et al., 2014). Finally, pH was not controlled during the whole fermentation process. Such fact allows to reduce the overall cost since there was no need for the use of reagents such as basis/acids or additional equipment (Tamis et al., 2015).

4.2.2. Results of acidogenic fermentation of HSSL

The acidogenic fermentation of HSSL in the CSTR1 system lasted 262 days. The SCOA distribution in terms of general composition, the SCOA and substrate concentrations and the variation of pH in CSTR1 are represented in Figure 11. The HRT initially chosen was 2.34 days (HRT1), with the pumps working at a flow rate of 0.85 L/day. The variation in SCOA production and sugars consumption verified until day 181 indicated that despite the culture robustness associated with the capacity to survive and use a complex substrate such HSSL as carbon source, the culture was incapable to maintain the balance of AF process. The sugars were not totally consumed and the high variation in SCOA production and the SCOA concentrations obtained were rather low when compared to the results obtained previously (Queirós et al., Submitted). Queirós et al., (Submitted) achieved a maximum SCOA concentration of 7.45 gCOD/L after the stabilization of the system was reached. In the present study, a maximum concentration of 6.37 gCOD/L was obtained for HRT1 (Table 6), however for this value the system was unstable and the SCOA concentrations for the rest of the HRT1 fermentation time were quite low. This happened, probably, due to the fact that the culture used as inoculum was stored at 4º C for three months before the inoculation, which may have led to partial loss of acidogenic microorganisms. The age of the inoculum and the fact that it was stored for so long can be a possibility to explain the results obtained for the CSTR1 which showed quite unstable at HRT1. Also, the literature reports that longer HRT usually favors the AF (Lee et al., 2014), consequently an increase of HRT from 2.34 days (HRT1) to 3.01 days (HRT2) was applied by decreasing the feeding rate from 0.85 L/day to 0.66 L/day and consequently the OLR from 7.62 gCOD/L·d to 5.95 gCOD/L·d. In this way, sugars consumption increased and also higher SCOA concentrations were obtained along with the stabilization of the system (Figure 11). For instance, while at HRT1 the average SCOA concentration was 3.10 gCOD/L, when the HRT2 was applied, the average value achieved was 3.53 gCOD/L. Most important than higher SCOA concentrations is the equilibrium of the system, which showed to be more

stable at HRT2. In summary, HRT1 was applied during the first 181 days, from which was changed to HRT2 during the last 81 days of CSTR1 operation.

The first 35 days are described by a major instability in the SCOA production and sugars consumption. Also, it is notable the increase of pH between the days 17 and 21, which was caused by the addition of a new feed whose pH was not adjusted to 6.0 as it should, thus causing a major increase of the pH of the reactor. This variation in the pH showed a considerable impact on the SCOA produced, since acetic acid stopped being produced until the system pH was reestablished. In the following days an increase in the SCOA concentration was observed until day 28 reaching a maximum of 6.37 gCOD/L. After this day SCOA concentrations lowered to values of almost half of this maximum.

Due to the system complexity, as a result of a complex substrate and the use of a MMC, a stationary phase could not be achieved, with a stable concentration of SCOA. Thus, a pseudo-stationary phase (PSS) was defined as a stage of the process during which the system remained relatively stable, with small variations in the SCOA produced. The PSS1 for HRT1, in this case, was achieved from the day 41 to 148. During this time, the SCOA concentrations suffered small variations After the HRT change HRT2, the system took only 15 days to reestablish the balance and a PSS2 was achieved at day 196.

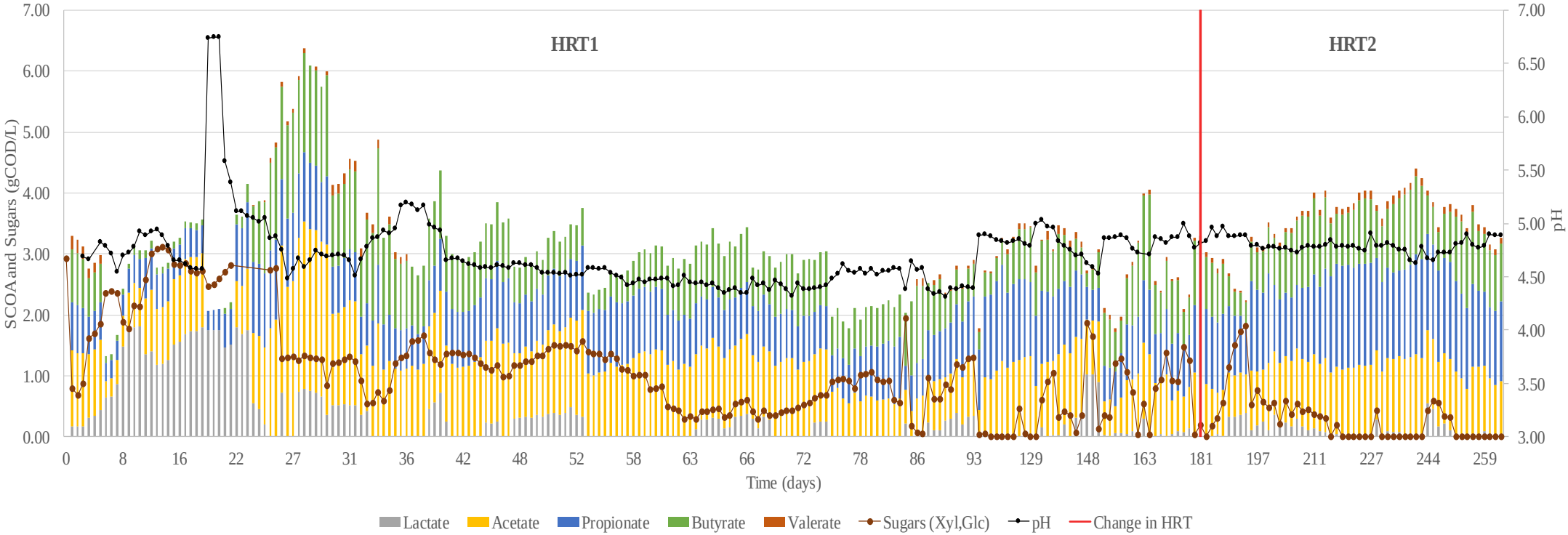

Figure 11. Distribution of SCOA and its variation, along with the substrate (xylose and glucose) and pH variation over the fermentation time for CSTR1

The consumption of xylose and glucose was not simultaneous. During the instability period, before PSS1, the sugars consumption was not stable and there was a simultaneous consumption of xylose and glucose. After the PSS1 was achieved, glucose started being consumed preferentially and its concentration was kept stable at values near zero (below 0.10 gCOD/L) for the rest of the time (Figure 12). On the other hand, xylose consumption was quite unstable until the PSS2 was achieved, at day 196. After this day, xylose started being fully converted, presenting concentrations of zero or near (below 0.50 gCOD/L).

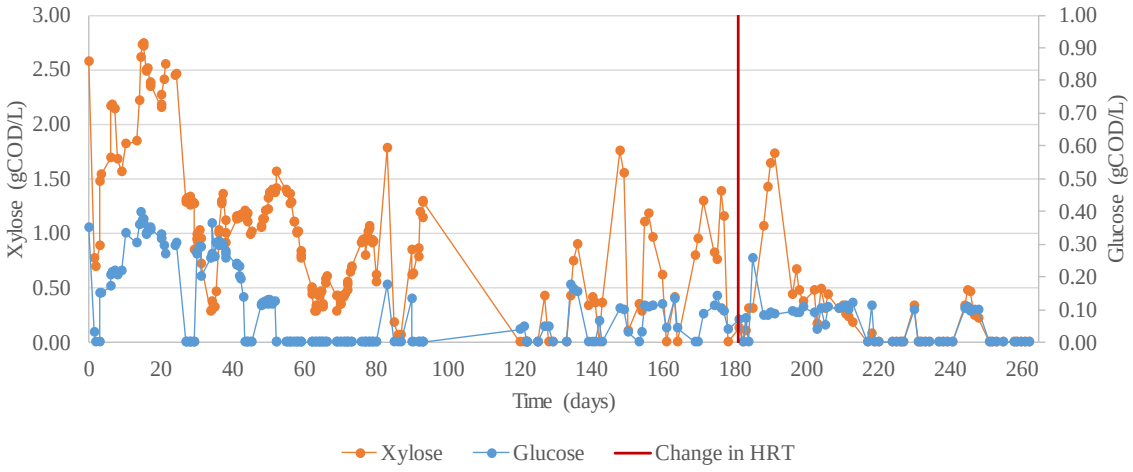

Figure 12. Variation of xylose and glucose concentrations (gCOD/L) during the fermentation time.

The preferential consumption of glucose was expected due to its direct biochemical pathway. While glucose monomer is readily metabolized in glycolysis (Figure 4), xylose needs a first step of conversion to the intermediary D-xylulose-5-phospate, which is then metabolized by either the pentose-phosphate pathway or by the phosphoketolase pathway, as can be seen in Figure 5 (Jeffries, 1983; Temudo et al., 2009). The sequential use of these substrates was already reported by Queirós et al., (Submitted) using the same substrate, HSSL.

Sugars were only totally consumed for some small periods of the fermentation time (Figure 11 and Figure 12). The shorter HRT applied at the beginning was determinant for the inefficient consumption of sugars. With HRT2, sugars were more efficiently consumed. Also, the difficulty verified by the MMC to metabolize sugars probably was associated with the low pH values experienced in the reactor, since some studies indicate that low pH values have a negative impact in the conversion and growth rates of anaerobic fermentations (Tamis

et al., 2015; Temudo et al., 2007; Yu and Fang, 2002). Thus, the low SCOA concentrations achieved in the CSTR1 may also be associated to the reactor pH.

The peak of sugars concentration seen close to day 86 was simultaneous with a slight drop of pH values (Figure 11). A similar peak was verified close to day 148 and was also simultaneous with a drop in pH. Again, the decrease of pH was associated with problems in sugars consumption. At day 86 the peak of sugars was followed by its quick consumption to values near zero, which was possibly associated with the increase of pH. This same tendency was verified for the days 31, 93 and 148. It is noteworthy that this inverse relation between the sugars consumption and the decrease or increase of pH was only verified for pH values close to 4.50, indicating that this pH value might represent a limitation for the cultures. When the pH values dropped quickly to values close to 4.50, the sugars consumption was considerable affected and was followed by an improvement in the sugars consumption when the pH increased. After the HRT2 was applied and the PSS2 was achieved, the sugars concentrations presented a more stable profile, with small variations when compared to those observed earlier in the fermentation.

The first days of fermentation, until PSS1, were characterized by a significant production of lactic acid. Between days 17 and 21, in which the pH increased, lactic acid was the only SCOA who kept its concentration stable. This may indicate that the production of lactic acid is associated with instability phases or perturbations of the system, like the start of the process or modifications on the fermentation parameters as observed by Temudo et al., (2007). Duque et al., (2014) also observed this pattern of lactate production in adaptation stages and temperature perturbations in the acidification of cheese whey and sugarcane molasses. During the PSS, lactic acid was produced in minor amounts, and its production was probably due to small variations in the system. These include small drops in the pH, since it is known that lower pH values benefit the production of lactate (Itoh et al., 2012; Wu et al., 2015).

Despite pH was not controlled, it remained quite stable during the whole process, with an initial value of 4.69 and a final value of 4.77. This can be attributed to the continuous mode of operation, that prevents the accumulation of SCOA in the system, and to the buffer capacity of HSSL, previously reported by some authors (Cruz, 2014; Queirós et al., 2014). These factors prevented the drop of pH to inhibitory values for the culture, being an advantage to the system as referred earlier. Also, the low pH values verified in the CSTR1

also inhibit methanogenic microorganisms possible existent in the reactor. The use of aerobic cultures herewith low pH values is an effective way to inhibit methanogenesis.

The maximum concentration of SCOA achieved was 6.37 gCOD/L at day 28 of fermentation process, during the HRT1 operation, and this value corresponded also to the maximum AD_{total} of 35.7% and AD_{sugars} of 217%. The HRT2 operation, on the other hand, presented the highest SCOA concentration of 4.40 gCOD/L, corresponding to the maximum AD_{total} of 24.7% and AD_{sugars} of 150%. AD_{sugars} was calculated taking into account only the sugars (xylose and glucose) consumption while AD_{total} was calculated considering the consumption of the whole organic matter available as substrate.

The average value of AD_{sugars} achieved for HRT1 was 106% and for HRT2 was 121%. These values, along with the maximum values presented above, indicate that there were other components of the HSSL being consumed along with xylose and glucose namely other monomeric sugars such as ramnose, arabinose, mannose and galactose which together present a concentration of 7.06 gCOD/L in the HSSL pre-treated (data provided by Caima) and whose concentration in the samples could not be evaluated. In addition to that, it is important to note that a significant part of dissolved carbohydrates (up to 25%) is present in HSSL as oligosaccharides (Pereira et al., 2013; Xavier et al., 2010), which can possible be hydrolyzed into its monomers and converted to SCOA, thus explaining the yield higher than 100%. With this in mind, for comparison with the literature, only the AD_{total} was considered. The $Y_{SCOA/Sugars}$ achieved for this system was 1.75 gCOD/gCOD for HRT1 and 1.35 gCOD/gCOD for HRT2, which is consistent with what was previously described about other sugars besides glucose and xylose being consumed and with the AD_{sugars} calculated. In the case of the whole organic matter present, the $Y_{SCOA/S}$ was calculated and the values of 0.78 gCOD/gCOD and 0.89 gCOD/gCOD were obtained for HRT1 and HRT2, respectively. If an average AD_{total} was considered, 17.4% and 19.8% were achieved for HRT1 and HRT2, which was significantly below the maximum AD reported above for CSTR1. This can be explained by the instability of the system, that had higher SCOA concentrations in the first 30 days of fermentation, followed by a decrease in SCOA production for the rest of fermentation time. The average SCOA produced was 3.10 gCOD/L for HRT1 and 3.53 gCOD/L for HRT2, less than half the maximum SCOA achieved in the day 28, during the first retention time applied. The results for the average AD_{total} achieved were lower when compared to the literature. For instance, Arroja et al., (2012) achieved an AD ranging from

30-65% for four different substrates used, being them sugarcane molasses, spent coffee grounds, dairy processing fatty slurry and cheese whey. The authors considered that the type of waste, simultaneously with the operational parameters imposed, such as HRT, OLR, pH and temperature, had a major effect on the success of the acidification process, and consequently, the AD achieved (Arroja et al., 2012). Silva et al., (2013) tested eight different substrates (cheese whey, sugarcane molasses, organic fraction of municipal solid wastes, glycerol, soapy slurry, winery wastewater, olive mill effluent and landfill leachate) and achieved a maximum AD of 51.6% for cheese whey, which corresponded to a maximum SCOA of 3.37 gCOD/L, followed by an AD of 42.1% and maximum SCOA of 3.11 gCOD/L for sugarcane molasses. Despite the AD values achieved by the authors are higher than those obtained in this work, if the maximum SCOA achieved is considered, the value obtained in this work, 6.37 gCOD/L, is quite higher than the values for all the eight substrates tested. Considering the average SCOA concentration achieved for HRT2, 3.53 gCOD/L, this value is still higher than those obtained by the authors in eight effluents tested. Also, the average AD_{total} achieved for CSTR1 at HRT2 was higher those from five of the effluents tested.

Bengtsson et al., (2008) also tested four potential wastewaters (cheese whey permeate and three pulp and paper mill effluents) for the production of SCOA in batch experiments and concluded that the maximum AD achieved and the time needed to accomplish it varied between the different wastewaters. The maximum AD achieved for that work were 67% for whey permeate and 66% for an effluent from a paper mill, after 8 and 11 days, respectively (Bengtsson et al., 2008). Also, authors showed that after this maximum was achieved, SCOA concentrations remained constant or with slight variations, reaching a PSS. The stabilization after achieving a maximum SCOA did not happen in this case for the both HRT values applied. In the current work, after achieving the maximum SCOA value in the 28º day, the production of SCOA decreased significantly and remained at lower values for the rest of fermentation time. The exception was verified after applying the HRT2, when the SCOA concentrations increased to values close to 4 gCOD/L. In terms of SCOA concentration, Bengtsson et al., (2008) achieved a maximum value of 3.96 gCOD/L SCOA in the end of the fermentation of paper mill wastewater, value not considered high when compared to the average value of 3.53 gCOD/L achieved in this case for the CSTR1 with HRT2. Taking into account that the authors tested the effluents acidogenesis in a batch fermentation, where the

SCOA accumulate, the concentrations achieved at the end of the experiment must be consequently higher than in a continuous operation.

The results achieved are significant when compared to the literature presented (Table 3) and considering the composition of HSSL, rich in recalcitrant compounds which are hardly biodegradable, and also rich in some microbial inhibitors. Thus, the results accomplished indicate that despite the HSSL composition, an efficient SCOA production process can be carried out. HSSL presents itself as a candidate to be valorized in a biorefinery plant in the future.

Table 3. Review of some of the results achieved for AF processes with different substrates performed at different conditions in comparison with the results of the present study. The SCOA profiles are presented in the order lactate, acetate, propionate, butyrate and valerate.

Substrate	Composition	System	HRT	OLR	Temperature (ºC)	pH	SCOA	SCOA Profiles (%)	Reference
Sugar cane molasses	Mainly sucrose and fructose	CSTR 1.14 L	10 h	35|31|37 Cmmol/L·h	30	5|6|7	238|194|209 Cmmol/L	3/36/14/28/22 | 0/63/17/14/7 | 7/50/26/11/5	Albuquerque et al., 2007
Food waste (simulated)	Rice 35%, cabbage 45%, pork 16%, tofu 4%-	Batch 4.5 L	8 d	-	35	Uncontrolled |5|6|7	3.94|17.1|39.5| 37.1 g/L	0/67.0/3.7/29.4/0 | 0/60.4/8.3/31.1/0.15 | 0/23.8/13.5/53.3/9.5 | 0/34.1/19.7/42.7/3.6	Jiang et al., 2013
Food waste (synthetic)	Glucose	Semi-continuous 2 L	8 d	9 g/L·d	35	5|5.5|6	18.0|24.0|25.5 g/L	0/18.2/4.4/18.4/0 | 0/33/28.1/21.6/14.3 | 0/50.9/25.1/21.5/7.0	Lim et al., 2008
Cheese whey	Lactose 78.4%, proteins 13.6%, fats 1.2%	CSTR + membrane filtration module 1.14 L	1 d	13.7 gCOD/L·d	37	6	9.7 gCOD/L	4/61/13/12/1	Duque et al., 2014
Sugar cane molasses	Sugars 54%			16.3 gCOD/L·d			13.2 gCOD/L	0/24/34/16/24	
Cheese whey	Lactose 78.4%, proteins 13.6%, fats 1.2%	CSTR 1.25 L	1 d	15.9 gCOD/L·d	30	4.5|5|6|7	11.0|14.1|13.7| 12.7 gCOD/L	80/18/0/1/0 | 67/30/2/0/0 | 48/40/10/2/0 | 56/37/6/0/0	Gouveia et al., 2016
	LS 70.7%, xylose 14.4%, glucose 2%	CSTR 1.55 L	1.76 d	11.8 gCOD/L·d	30	Uncontrolled	5.50 gCOD/L	5.7/53.6/22.0/18.7/0	Queirós et al., (Submitted)
HSSL	LS 70.7%, xylose 14.4%, glucose 2%	CSTR 2 L	2.34|3 d	7.62|5.95 gCOD/L·d	30	Uncontrolled	3.10|3.53 gCOD/L	4.43/36.7/32.1/26.0/0.86 | 2.90/29.9/38.3/26.2/2.73	Present study
			3	5.95 gCOD/L·d		6|7|8	2.36|2.38|2.27 gCOD/L	0.01/57.8/27.0/14.8/0.36 | 1.32/82.7/1.28/14.9/0.11 | 1.55/89.2/1.88/7.20/0.26	
		MBBR 3.22 L				Uncontrolled	2.32 gCOD/L	0.00/23.0/15.9/59.9/1.21	

4.2.3. SCOA distribution

The SCOA profiles were calculated taking into account only the SCOA produced, which means that the acetic present in the HSSL was not considered. Considering the HRT1 and PSS1, the SCOA profile obtained was 4.43/36.7/32.1/26.0/0.86 % for lactate, acetate, propionate, butyrate and valerate, respectively. If the HRT2 and PSS2 were considered, the profile achieved was 2.90/29.9/38.3/26.2/2.73 %. At HRT1, although acetate was the main SCOA produced, propionate also showed a high percentage, followed by butyrate. In the case of HRT2, propionate was the main SCOA produced, followed by acetate and butyrate, respectively. Also, at HRT2 the percentage of valerate present increased.

In general, the main SCOA produced were acetic, propionic and butyric acids, which was in agreement with previous results reported (Bengtsson et al., 2008; Jiang et al., 2013; Jie et al., 2014; Yu and Fang, 2002). Iso-butyric acid was not produced during the experiment. Lactic acid was produced during the instability stages, in higher concentrations during the start of the fermentation and then in smaller amounts during the rest of the process. Also, valeric acid was produced almost in negligible concentrations with an average of 0.86% and 2.73% for HRT1 and HRT2, respectively. This was expected since valerate is not normally produced in this type of process or produced in insignificant amounts (Jankowska et al., 2015; Temudo et al., 2007; Yu and Fang, 2002). Some authors stated that while acetic, propionic and butyric acids can be produced directly from the fermentation of soluble proteins, carbohydrates and lipids, valeric acid is mainly produced from proteins degradation (Wang et al., 2014; Yu and Fang, 2002). Taking into account that the HSSL has no protein content, the production of valeric acid is highly improbable in this case. This can be related to the length of valeric acid chain, with five carbon atoms, which probably is not so favorable for the cultures to synthesize. Furthermore, Liang and Wan, (2015) used brewer's spent grain as substrate for SCOA production and demonstrated that under neutral conditions lactic acid and ethanol were consumed and related that with the accumulation of SCOA. Thus, Liang and Wan, (2015) studied the addition of lactate to the system and observed that the lactate consumption was related mainly to the increase of propionic and butyric concentrations, but also with the production of valeric and caproic acids in smaller amounts. Thus, authors considered that although carbohydrates are the main source of carbon to SCOA production, lactic acid can also be used as electron donor to produce SCOA. Although the pH

experienced on CSTR1 was not neutral and the substrate was different, this can be an explanation for the production of valeric acid which normally occurred simultaneously with lactic acid production or right after these periods (Figure 11).

As verified by Queirós et al., (Submitted), it was notorious a shift between the propionic and butyric acids concentrations (Figure 13). This shift was already observed by Cohen et al., (1984) which tested the acidogenic fermentation of a synthetic medium and showed that when a higher butyrate production was achieved, a lower formation of propionate was found and vice versa. These authors also reported that a high production of butyrate correlated positively with the production of hydrogen and carbon dioxide, while a high production of propionate correlated negatively, indicating that when propionate was mainly produced, there was little or none production of gas. In addition to this, it was also stated that the production of acetate was high in both occasions, thus showing no correlation to the production of both butyrate and propionate. The same tendency was verified by Bengtsson et al., (2008) for whey and paper mill wastewaters while testing different retention times and for whey while testing different pH values. The shift between the production of propionate and butyrate can be explained by the competition existent between propionate fermentation type (that produces mainly acetate and propionate) and butyrate fermentation type (which produces mainly acetate and butyrate) (Bengtsson et al., 2008; Cohen et al., 1984; Horiuchi et al., 2002). Since the two types of fermentation are carried out by different groups of microorganisms, it is possible that any change in the reactor may cause the dominance of one group, and vice versa, thus associated with the production of propionate or butyrate inversely. Also, just like Cohen et al., (1984) verified and as can be seen, acetate production does not appear to be correlated with propionate and butyrate production in particular (Figure 13).

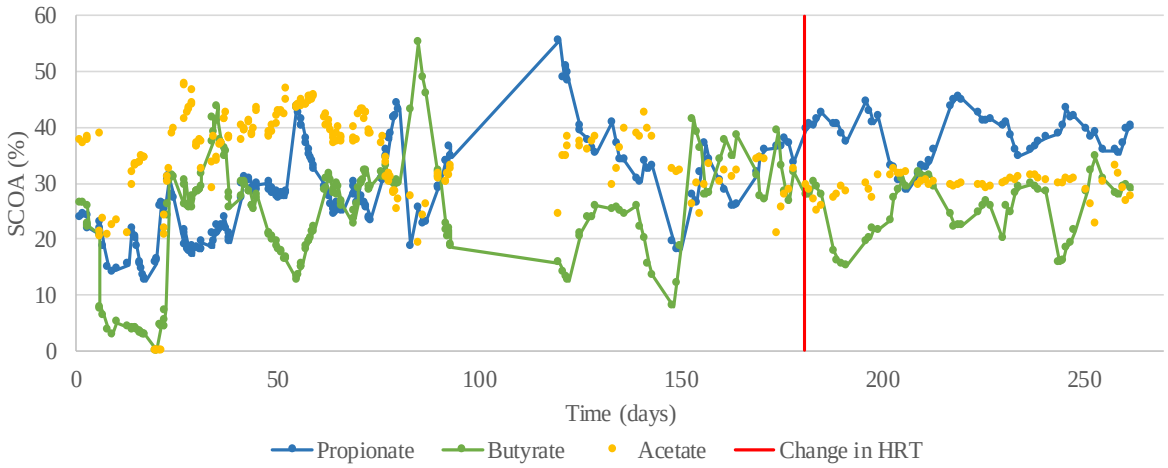

Figure 13. Representation of the acetate variation along with the inverse variation of propionate and butyrate, in % calculated from gCOD/L, in the CSTR1 along with the fermentation time.

Considering the effluent composition, therefore using the total acetate present in the samples to calculate the percentages, the profile obtained was 3.57/53.5/23.7/19.3/0.62 % for HRT1 and 2.50/44.5/30.1/20.4/2.14 % for HRT2, for each PSS. The percentages of 23.7% and 30.1% achieved for propionate are crucial taking into account the downstream of the process – the production of PHA. As said before, in PHA production process, the types of SCOA used as substrate present an extreme importance to the polymer composition. Thus, the higher the percentage of propionate in the effluent stream used to feed the selection and accumulation sequencing batch reactor, the higher the content in HV of the polymer, which improves the characteristics and commercial value of PHA (Queirós et al., 2014).

In addition to the SCOA concentration, also the LS and COD of the CSTR1 were evaluated during the first 136 days. As can be seen in the Figure 14, the variation of LS is in accordance with the COD variation. The increase of COD, and in consequence of LS, in the first days of fermentation occurred due to the renewal of the medium inside the reactor since new feed was being added to the culture.

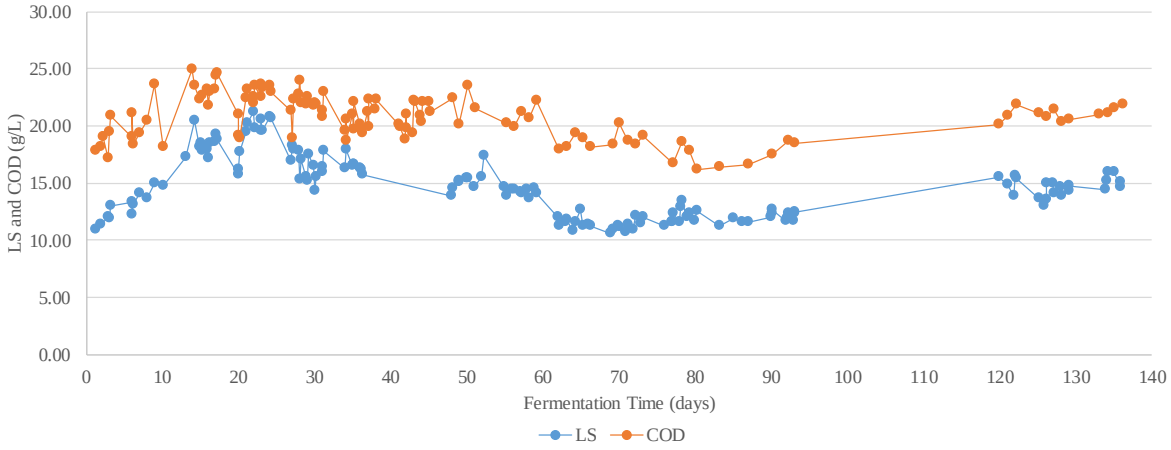

Figure 14. Variation of LS and COD, in g/L, over the first 136 days of fermentation time in the CSTR1.

The AF process is an intermediary stage in which the products achieved, SCOA, are also organic compounds and can be used as carbon source by microorganisms. The amount of sugars consumed is being converted in the same amount of SCOA, considering the COD units using the conversion factors (Dogan and Demirer, 2009). Thus, it was not necessary to control so roughly the COD variation of the process such as in a process using insoluble/solids substrates, in which the soluble COD increase during the fermentation (Maspolim et al., 2015; Yin et al., 2016). In this study, COD started to be monitored, regularly, after the day 136. Besides, since the production of gaseous products was not significant due to the inhibition of acetogenesis and methanogenesis, the soluble COD did not decrease (Dahiya et al., 2015). On the other hand, it was important to monitor the COD of the feed in order to verify possible errors in the feed preparation and also to control the variations of the feed. Moreover, a more efficient way to perform this monitoring would be by the separate addition of the mineral medium and the HSSL.

Since the LS variation followed the COD variation, it can be concluded that the variations of LS verified occurred due to the variation of the system COD. Notwithstanding, from these results it was concluded that the LS were not being consumed. After the 136º day its quantification was made regularly to effects of control.

4.2.4. Biomass variation

The biomass concentration was evaluated for all the fermentation time and its variation is represented in Figure 15. As can be observed, during the whole fermentation time the

biomass concentration suffered many variations, even after the PSS was reached. In comparison with Figure 11, it can be concluded that biomass variation did not seem to have a relation with the SCOA production. Although the concentration of biomass does not seem to affect SCOA production, the species present might have a crucial role in the AF process and the types of SCOA produced (Maspolim et al., 2015; Temudo et al., 2008). Thus, the evaluation and identification of the culture present in the reactor would be important in order to stablish a relation between the species and the SCOA variations in the CSTR1.

The minimum value of biomass reached was 0.95 g/L for the day 176, while the maximum was 5.04 for the 20º day. The variations of biomass were recurrent and could be attributed to batch stages, small perturbations of the system (e.g. electric failures, contaminations), among others. Despite that, biomass concentration only was below 1 g/L for its minimum, which indicates that there was no risk of washout. This shows that despite the culture was resistant and robust, it was sensitive to small variations in the system.

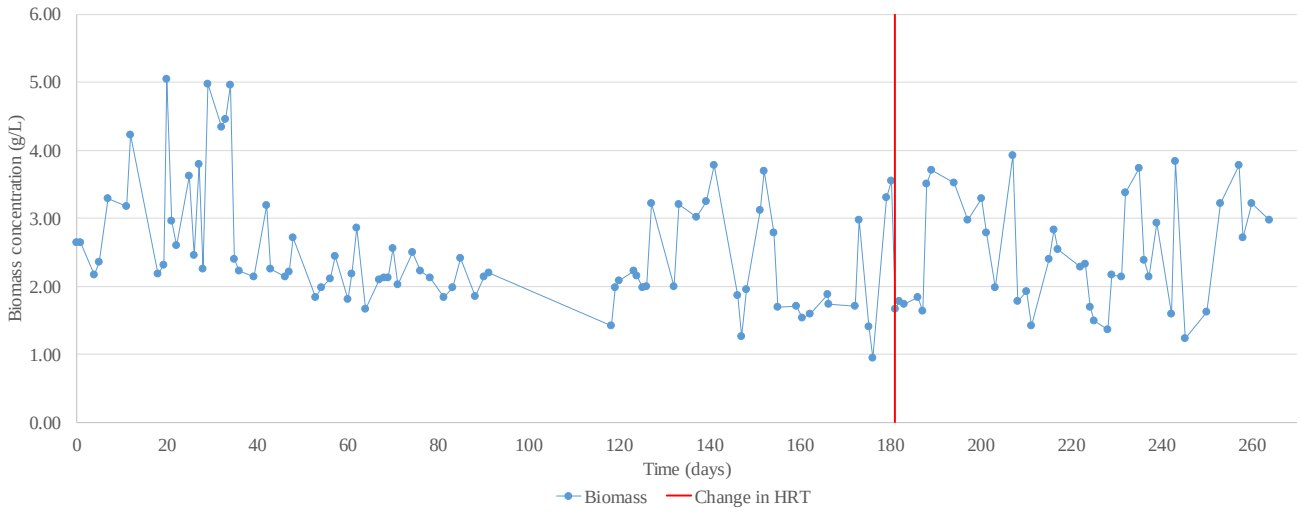

Figure 15. Variation of biomass concentration, in g/L, during the fermentation time in the CSTR1.

4.3. Effect of initial pH

4.3.1. Choice of the conditions imposed

A set of batch experiments had as main objective the observation of the influence of the initial pH on the AF of HSSL. These tests were performed for two different inocula, one with the acclimatized sludge (AS) from the CSTR1 at HRT1, with a concentration of 1.65

g/L, and the other with fresh sludge (FS) from WWTP, with a concentration of 11.9 g/L. The use of two different populations as inocula had as main objective the study of its behavior when subjected to different initial pH in terms of SCOA production and acidogenesis stability.

Conditions such as the fermentation medium, COD concentration of HSSL and temperature used were chosen accordingly to the parameters applied in CSTR1. In order to achieve the required pH value, appropriate buffer solutions were added to the flasks.

4.3.2. Overall results

The batch experiments lasted 25 days for AS and 28 days for FS. Due the accumulation of SCOA in the flasks, the initial pH of all the tests dropped quickly after the first day of fermentation, as can be seen in the graphics presented on Appendices A and B. This effect showed a major impact in the experiments with high pH initial values, such as pH 8 and 9. In addition to that, in some cases, the pH suffered considerable fluctuations during the fermentation time.

The graphics presented in the Appendices A and B show the SCOA distribution and variation, along with the variation of the substrate and pH. Also, Table 4 and Table 5 present the consumed substrate, SCOA produced in the end of the batch test, yield of SCOA produced from sugars $Y_{(SCOA/Sugars)}$, xylose and glucose, and from all the organic matter available $Y_{(SCOA/S)}$, AD_{sugars}, AD_{total}, substrate and product volumetric rates ($-r_s$ and r_p) and the SCOA profiles achieved, for the whole fermentation time and for the PSS.

As can be observed, the batch experiments with FS as inoculum achieved better results. For instance, for the tests with the inoculum with the AS the maximum SCOA produced in the end of the fermentation was 7.46 gCOD/L for pH 7 (with pH stable slightly above 6), while for the other set of experiments, with the FS, the maximum SCOA achieved was 9.11 gCOD/L for pH 5 (with pH stable slightly below 6). The FS demonstrated to be more effective when it comes to SCOA production, yields and AD_{sugars} and AD_{total} in most the pH tests, with the exception of pH 7 and 9, in which the results were slightly lower than those obtained for the other set of experiments. While AS presented 47.0% as the higher AD_{total} for pH 7, FS presented AD_{total} higher than 50% for pH 5 (57.9%) and pH 6 (53.1%), which was remarkable due the composition of HSSL, with a low concentration of sugars when compared with other compounds such as LS and extractives, that are not normally consumed

due to their recalcitrance and inhibitory effects (Pereira et al., 2013). Taking into consideration the overall results, FS was chosen to inoculate the CSTR2 and test the different pH values. Since the higher SCOA production, yields and AD were obtained for pH 5 and 6 batch tests and in the first one the pH was above 5.5 during almost all the experiment, the pH 6 was chosen to start the CSTR2. Wang et al., (2014) also achieved the higher SCOA production for pH 6, results similar to Jiang et al., (2013) and Lim et al., (2008), all of them using food waste as carbon source.

Table 4. Summary of the results from the batch tests with the AS from the CSTR1, namely the consumed substrate, SCOA produced, yields, volumetric rates, AD and SCOA profiles. The SCOA profiles were calculated as an average of all the fermentation time and PSS.

AS batch Test (pH)	Consumed substrate (gCOD/L)	SCOA produced (gCOD/L)	$Y_{SCOA/Sugars}$ (gCOD/gCOD)	$Y_{SCOA/S}$ (gCOD/gCOD)	$-r_s$ (gCOD/L·h)	r_p (gCOD/L·h)	AD_{sugars} (%)	AD_{total} (%)	SCOA profile Lactate/Acetate/Propionate/Butyrate/Valerate (%)	SCOA PSS profile Lactate/Acetate/Propionate/Butyrate/Valerate (%)
4	1.20	0.00	0.00	0.00	0.0020	0.00	0.00	0.00	-	-
5	1.27	2.63	2.07	0.17	0.0021	0.0044	207	16.6	37.2/30.5/13.6/12.06/0.38	39.8/35.4/13.2/11.7/0.00
6	3.34	5.96	1.78	0.38	0.0056	0.010	178	37.6	0.73/41.1/24.5/27.2/0.26	0.00/51.1/27.7/21.2/0.00
7	3.34	7.46	2.23	0.47	0.0056	0.013	223	47.0	3.41/50.1/18.7/20.7/0.79	0.00/60.2/18.4/21.0/0.51
8	3.34	3.68	1.10	0.23	0.0056	0.0062	110	23.2	4.70/57.7/16.2/15.1/0.14	0.00/65.3/17.6/17.1/0.00
9	3.34	5.00	1.50	0.32	0.0056	0.0084	150	31.5	5.16/54.4/15.0/19.2/0.00	0.00/50.4/15.7/33.9/0.00

Table 5. Summary of the results from the batch tests with the FS, namely the consumed substrate, SCOA produced, yields, volumetric rates, AD and SCOA profiles. The SCOA profiles were calculated as an average of all the fermentation time and PSS.

FS batch Test (pH)	Consumed substrate (gCOD/L)	SCOA produced (gCOD/L)	$Y_{SCOA/Sugars}$ (gCOD/gCOD)	$Y_{SCOA/S}$ (gCOD/gCOD)	$-r_s$ (gCOD/L·h)	r_p (gCOD/L·h)	AD_{sugars} (%)	AD_{total} (%)	SCOA profile Lactate/Acetate/Propionate/Butyrate/Valerate (%)	SCOA PSS profile Lactate/Acetate/Propionate/Butyrate/Valerate (%)
4	1.15	0.36	0.31	0.02	0.0017	0.0005	31.0	2.27	-	-
5	3.77	9.11	2.42	0.58	0.0056	0.014	242	57.9	35.8/31.8/11.5/13.6/0.12	5.20/48.5/14.2/31.8/0.27
6	3.77	8.35	2.22	0.53	0.0056	0.012	222	53.1	15.8/46.9/14.5/14.5/1.21	2.82/55.8/18.6/21.0/1.88
7	3.77	5.87	1.56	0.37	0.0056	0.0088	156	37.3	5.98/59.4/14.2/12.8/0.41	1.23/65.4/16.1/16.6/0.63
8	3.77	4.17	1.11	0.26	0.0056	0.0062	111	26.5	4.21/57.8/16.5/13.9/0.51	1.00/64.9/16.2/17.2/0.80
9	3.77	4.70	1.25	0.30	0.0056	0.0070	125	29.9	5.38/56.9/21.6/8.91/0.08	2.60/64.6/23.4/9.28/0.11

The AD_{sugars} for all the experiments (excluding pH 4 batch tests) obtained was higher than 100%. The yields higher than 100% were explained earlier for CSTR1 results and are related to the consumption of other components of HSSL, probably other monomeric sugars that were not quantified. For AS the highest AD_{sugars} was 223% for pH 7 and for FS was 242% for pH 5.

Also, FS demonstrated a minor variation between the AD_{total} obtained for the six pH values tested. These results showed that FS, a culture without acidogenic microorganisms selected, was more effective in SCOA production when subjected to different initial pH values. This was not expected since AS was already rich in acidogenic microorganisms but adapted to CSTR1 conditions. These results are discrepant with those achieved by Arroja et al., (2012) that also tested two different inocula, one with pre-acclimatized anaerobic sludge for SCOA production and other with conventional mixed anaerobic sludge, in two MBBR systems. These authors reported a much higher AD with the pre-acclimatized sludge.

Both AS and FS experiments showed the poorest results at pH 4. In the last day of the experiments, AS had no SCOA produced and FS presented 0.36 gCOD/L of SCOA. Although there were none SCOA produced at the end of fermentation time in the AS pH 4 test, the Figure 21 in the Appendix A shows that in the first seven days of acidification a small amount of SCOA was produced, which can be enlightened by the fact that AS was already adapted to the low pH values verified in the CSTR1. Besides pH 4 tests, the yields and AD of the other experiments were reasonably stable between them. Gouveia et al., (2016) also tested some pH values for the AF of cheese whey and obtained a similar result, with lower fermentation yields for pH 4. Authors considered that pH had a major impact in the acidogenic populations and metabolism. Besides, Jankowska et al., (2015) stated that pH affects the growth rate, the utilization of the carbon source and the efficiency of substrate conversion. Also, Yu and Fang, (2002) reported that the degradation efficiency of carbohydrate was pH sensitive for pH values less than 5.5 using dairy wastewaters as substrate. Lower pH causing a drop in SCOA production was also verified by Bengtsson et al., (2008) which used cheese whey and an effluent from a paper mill as carbon source. These authors showed that the SCOA production was significantly reduced below pH 5 for whey and below pH 5.5 for the paper mill effluent.

In almost all the experiments lactate was produced at the initial stages of the fermentation, which again indicates that the production of this acid may be associated with

perturbations of the system. Besides pH 4 experiments, where the culture was clearly inhibited, higher concentrations of lactate were observed at pH < 5 (Figure 22, Appendix A), in which pH was nearly constant at 4.5 and lactate was produced during all the fermentation time at significant concentrations. This was not observed for pH 5 experiment using FS since the pH values in this test were maintained above 5. All these results were in accordance with some authors that evidenced a higher lactate production at low pH values due to the dominance of some lactic acid bacteria which are more resistant to extreme conditions such as acidic environments (Gouveia et al., 2016; Itoh et al., 2012; Queirós et al., Submitted; Wu et al., 2015). These bacteria can produce antimicrobial substances along with lactate which can inhibit the growth of SCOA producing microorganisms. Furthermore, these results are also concordant with what was referrer earlier, that lactate can be used as substrate to produce other SCOA at neutral pH values (Liang and Wan, 2015).

The stability of the process may be associated with the stability of pH, since for some tests the stabilization of SCOA concentrations and the total consumption of the substrate were simultaneous with the stabilization of the pH. The fact that pH variations have a major impact in SCOA profiles and concentrations was observed for pH 9 test with AS, in which the variation of pH values is consistent with variations in the fermentation products and concentrations.

4.3.3. SCOA distribution

The average SCOA and PSS profiles obtained are also resumed in Table 4 and Table 5. They were calculated taking into account only the amount of acetate produced in the AF. Although the acetate in the feed is important since in an implemented process of AF with this substrate, it will have a major contribution in the composition of the effluent, in this case it was not considered since the objective was to compare the production of the different SCOA for each batch test. The PSS was considered when the SCOA production and profiles became relatively stable. For both AS and FS experiments, the PSS was not achieved for pH 4 tests since there was nearly no SCOA production. The increase in SCOA concentration after sugars complete consumption observed in some experiments might indicate that other substrates, probably other monomeric sugars or even oligosaccharides dissolved in HSSL were hydrolyzed into monomers and consumed for SCOA production. Since HSSL is rich in LS, the concentration of these compounds was also evaluated for all the experiments.

Nevertheless, there was no LS consumption observed in the experiments, only some slight variations.

As can be seen, the average and PSS profiles differ considerable in the percentage of lactate for both the experiments which was already explained above by the fact that the production of lactate can be associated to perturbations of the system. Furthermore, the higher percentages of lactate achieved in PSS were at lower pH, which is in accordance with what was discussed earlier. Just like in the CSTR1, in the batch tests the main products were also acetic, propionic and butyric acids.

For the SCOA profiles of AS experiments (Table 4), considering the PSS, pH 5 presented the minimum percentages for acetate, propionate and butyrate. Lactate was only produced at this pH value, and was the main component, 39.8%. The percentage of acetate increased with the increase of pH until pH 8 (65.3%), while for pH 9 the percentage of acetate decreased to 50.4%. This acid was produced preferentially when compared to the other SCOA, as can be seen in the profiles obtained. The percentage of propionate had its minimum for pH 5, with 13.2%, and its maximum for pH 6, with 27.7%, and then decreased from pH 7 to 9. The decrease of propionate with pH increase is concordant with the results obtained by Yu and Fang, (2002), where it was verified an increase of propionate production, from 12% at pH 6 to 38% at pH 4 from dairy wastewaters. In this work the same tendency was verified, however the pH 5 showed lower propionate production, while pH 6 showed the highest propionate concentration. Such discrepancies can be explained by the use of a totally different carbon source. Considering butyrate, this acid also had its minimum for pH 5 (11.7%) and its maximum for pH 9 (33.9%), but no relation with pH values was observed. These results are discrepant when compared to those obtained by Jankowska et al., (2015), where it was verified that at an alkaline environment a lower percentage of butyrate was achieved, while for acidic environment noticeable amount of butyrate was detected. Valerate percentage was only observed in pH 7 experiment and it was residual, of 0.51%.

The profiles for FS (Table 5), also during PSS, were slightly different, with lactate being present in all the experiments, with percentages below 6% and its maximum for pH 5, in accordance with AS tests, with 5.20%. Acetate was, again, the main acid produced, with higher percentages than for AS, except for pH 8. The maximum percentage for acetate was achieved at pH 7 (65.4%) and the minimum at pH 5 (48.5%) and it was observed an increase of acetate percentage with the increase of pH. Just like AS tests, acetate was generally the

main acid produced. As can be seen in Table 3, Albuquerque et al., (2007) tested pH values of 5, 6 and 7 and likewise achieved a higher percentage of acetate for all the experiences, with acetate making up more than 50% of the SCOA produced. Furthermore, in the AS tests the acetate percentage increased with pH except for pH 9 in that case. These results were expected, since some authors verified this same tendency for the acetate production to increase with pH (Albuquerque et al., 2007; Lim et al., 2008). Propionate, on the other hand, had its minimum of 14.2% at pH 5 and its maximum for pH 9, 23.4%. However, it was not observed a connection between propionate variation and the increase of pH. For butyrate, this connection was perceived, since butyrate had as maximum 31.8% for pH 5, percentage that decreased with the increase of pH until 9.28% for pH 9. This result for butyrate is in accordance with the results obtained by Horiuchi et al., (2002), that achieved higher concentrations of butyrate for a pH range from 5 to 7, decreasing for pH 8 and are also concordant with the results achieved by Jankowska et al., (2015). Despite of that, these results are discrepant when compared to the ones obtained for AS, in which the shift in butyrate was the opposite. Lastly, for FS valerate was represented in all the experiments but once more with small percentages, with the maximum of 1.88% for pH 6. Iso-butyric acid was not produced during the both experiments.

Taking into account the difference between the lowest and highest pH values tested and the PSS, the SCOA profiles shifted from 39.8/35.4/13.2/11.7/0.00 % at pH 5 to 0.00/50.4/15.7/33.9/0.00 % at pH 9 for AS, for lactate, acetate, propionate, butyrate and valerate, respectively. For FS the shift was from 5.20/48.5/14.2/31.8/0.27 % to 2.60/64.6/23.4/9.28/0.11 %, from pH 5 to pH 9, respectively.

4.4. CSTR2

4.4.1. Choice of the operational conditions

The conditions imposed to this reactor were the same as those imposed to CSTR1, with the only difference of the pH control system and the HRT of 3.01 days being applied since the beginning. An OLR of 5.95 gCOD/L·d was imposed by the pumps working at a flowrate of 0.66 L/d, at a temperature of 30ºC. The inoculum used was fresh sludge that came from an aerobic tank, with a concentration of 11.6 g/L.

4.4.2. Results of acidogenic fermentation of HSSL

The objective of CSTR2 was to test the AF with same sludge used for FS tests since it was the one that achieved better results, along the pH value that also performed the best results in the batch experiments, pH 6. Despite the use of buffer solutions, the batch tests had some problems in maintaining the initial pH values and the pH drastically dropped for the higher pH values tested. Thus, the pH values of 7 and 8 were not evaluated correctly and were also chosen to be tested in the CSTR2. In this way, it was possible to evaluate the effect of pH on SCOA profiles and SCOA concentrations obtained in a CSTR system.

CSTR2 operated at pH 6 during 77 days, pH 7 during 35 days and pH 8 during 31 days. Overall, the CSTR2 worked during 143 days. The SCOA distribution and the SCOA and substrate variations in the reactor are shown in Figure 16.

As can be perceived, in contrast with the 41 days of instability before reaching PSS reported in the CSTR1, the system with pH control at 6 was stabilized quickly, being the PSS achieved at the day 17 after a gradual increase on SCOA production. Moreover, the PSS achieved for CSTR2 was more stable than the one for CSTR1, explained by the stability in the pH, which was already reported that has a strong influence in the process stability, plus the age of culture used (Horiuchi et al., 2002; Jankowska et al., 2015). After the PSS was reached in the first pH value tested, the SCOA concentrations remained quite stable even considering the pH variations from 6 to 7 and 7 to 8. Considering the whole fermentation time, after the PSS was achieved, the SCOA concentrations varied from a minimum of 2.04 gCOD/L for pH 8 to a maximum of 2.98 gCOD/L for pH 6, which prove the stability of the process when compared to the CSTR1 system. The PSS was also considered for pH 7 at day 102 and for pH 8 at the 129º day.

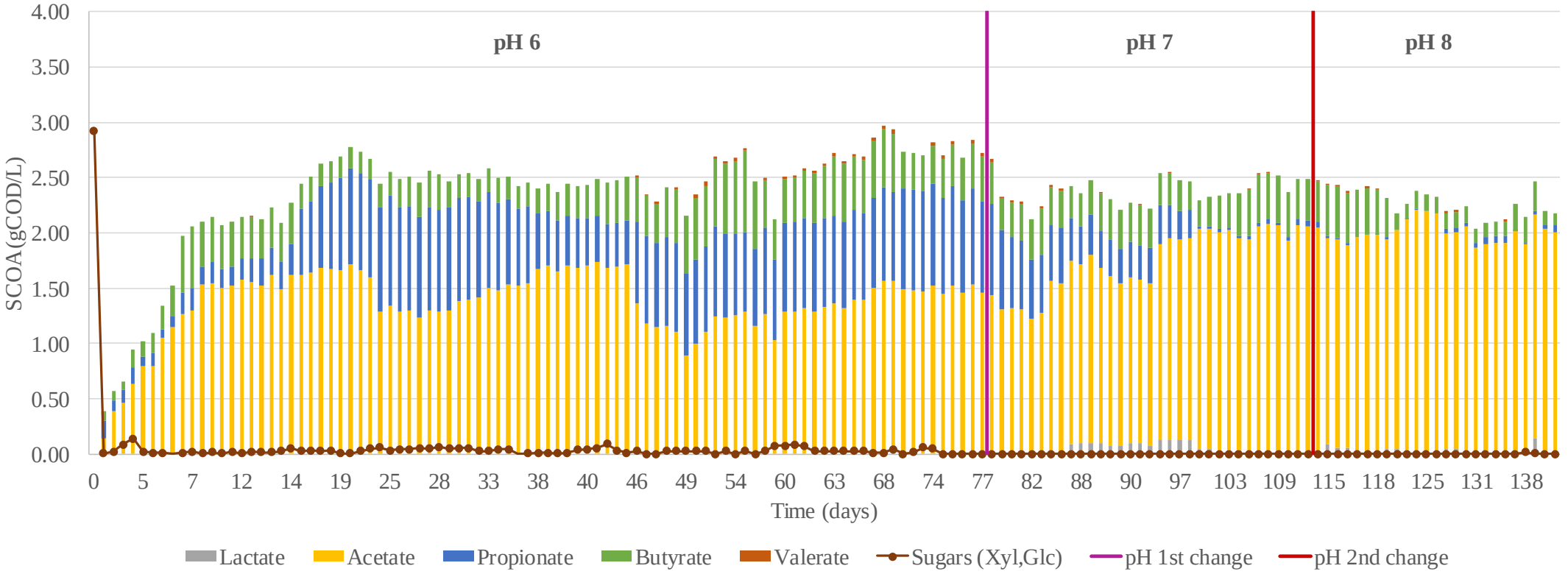

Figure 16. Distribution of SCOA and its variation, along with the substrate (xylose and glucose) variation over the fermentation time for CSTR2 for the three pH values tested.

Xylose and glucose present in the feed were almost totally consumed at pH 6, with residual concentrations during some periods, which did not occur for pH 7 and 8 in which the sugars were totally consumed. These results are in line with what was previously discussed for CSTR1 where the sugars were not completely consumed, fact that could be attributed to the low pH values of the system. Moreover, in the batch experiments, the increase of the consumption efficiency with the increase of pH was also observed.

Noteworthy, the residual production of lactic acid. This is in accordance with what was previously discussed about lactate being produced in instability phases and lower pH values. Since in this case the pH values tested were higher than 5 and the PSS was achieved quickly, without major variations, it was already expected the lactate production absence.

The maximum SCOA concentration achieved for pH 6 was 2.98 gCOD/L, for pH 7 was 2.67 gCOD/L and for pH 8 was 2.47 gCOD/L. These values corresponded to the maximum AD_{total} of 16.7%, 15.0% and 13.9%, respectively. All the values achieved were quite lower than the maximum values of 6.37 gCOD/L and 4.40 gCOD/L achieved for the CSTR1, that corresponded to the maximum AD_{total} of 35.7% and 24.7%, for HRT1 and HRT2, respectively (Table 6). Considering the average values, the maximum SCOA values obtained for CSTR2 were also lower than those achieved for the system without pH control, CSTR1, for the both HRT imposed. Considering the yields, the $Y_{SCOA/Sugars}$ achieved was higher for pH 6 and 7 with 0.82 gCOD/gCOD and 0.81 gCOD/gCOD versus the 0.78 gCOD/gCOD achieved for pH 8. When the whole organic matter available was taken into account, the $Y_{SCOA/S}$ was the same for the three pH values tested, 0.73 gCOD/gCOD.

Also, contrary to what was expected due to the results achieved for pH 6 in the batch experiments, when compared to the CSTR1 results at HRT2, the SCOA concentrations obtained for pH 6 in this system were lower. This is, consequently, followed by a low average AD_{total} of 13.2% when compared to the 19.8% obtained for CSTR1 at HRT2. Such observation could be explained by the deviation of the carbon source to the production, in considerable amounts, of other metabolites that were not identified due to lack of time. The peak of this metabolite in the HPLC is consistent with the peak of succinic acid in terms of retention time, fact that need to be proved in the future by a more accurate technique, such as HPLC coupled to a mass spectrometer (HPLC-MS). The production of succinate was already reported by Jankowska et al., (2015) from primary and waste activated sludge in which succinate was the dominant acid for pH 6 and retention time of 15 days, and by

Temudo et al., (2007) from glucose at a pH higher than 6. Also, Lim et al., (2008) reported the production of small amounts of succinate at pH 5.5 and major amounts of this same acid at pH 5, being succinate the main acid produced (44-48%) at this pH value, for the AF of food waste. In the case of this work, the production of this metabolite was only verified for pH 6 and not for pH 7 and 8.

Table 6. Summary of the results from the CSTR1 for HRT1 and HRT2, CSTR2 for pH 6, 7 and 8 and MBBR, namely the consumed substrate, SCOA produced, maximum SCOA produced, yields, volumetric rates, AD, the days that took to achieved PSS and SCOA PSS profiles. The consumed substrate, SCOA produced, yields, volumetric rates and AD were calculated as an average of all the fermentation time and the SCOA PSS profiles were calculated taking into account the PSS.

Reactor	HRT (days)	pH	Consumed Substrate (gCOD/L)	SCOA produced (gCOD/L)	Maximum SCOA produced (gCOD/L)	$Y_{SCOA/Sugars}$ (gCOD/ gCOD)	$Y_{SCOA/S}$ (gCOD/ gCOD)	$-r_s$ (gCOD/ L·h)	r_p (gCOD/ L·h)	AD_{sugars} (%)	AD_{total} (%)	Maximum AD_{total} (%)	PSS (day)	SCOA PSS profile Lactate/Acetate/Propionate/ Butyrate/Valerate (%)
CSTR1	2.34	-	1.77	3.10	6.37	1.75	0.78	0.025	0.043	106	17.4	35.7	41	4.43/36.7/32.1/26.0/0.86
	3	-	2.62	3.53	4.40	1.35	0.89	0.036	0.049	121	19.8	24.7	15	2.90/29.9/38.3/26.2/2.73
CSTR2	3	6	2.90	2.36	2.98	0.82	0.73	0.040	0.033	80.8	13.2	16.7	17	0.01/57.8/27.0/14.8/0.36
	3	7	2.93	2.38	2.67	0.81	0.73	0.040	0.033	81.6	13.3	15.0	25	1.32/82.7/1.28/14.9/0.11
	3	8	2.92	2.27	2.47	0.78	0.73	0.040	0.031	78.0	12.8	13.9	17	1.55/89.2/1.88/7.20/0.26
MBBR	3	-	2.71	2.32	2.85	0.86	0.73	0.037	0.032	79.5	13.0	16.0	26	0.00/23.0/15.9/59.9/1.21

4.4.3. SCOA distribution

Besides the different values of pH imposed to the CSTR2 did not seem to affect the SCOA overall concentration in a major extent, they had an effect on the SCOA distribution. While the SCOA distribution remained relatively stable during the operation time at pH 6, the pH 7 operation was marked by the appearance of lactate in small amounts along with the decrease of propionate. At pH 8, propionate and butyrate were produced in very low amounts, while the acetate concentration remained quite stable.

Once more, acetic, propionic and butyric acids were the main SCOA produced. Lactate and valerate were produced in residual amounts considering the overall process. Also, iso-butyric acid was not produced during the experiment. Butyric acid production remained relatively stable between pH 6 and 7, 14.8% and 14.9%. However, it decreased during the operation at pH 8 to 7.20%. The results achieved are in line with the study performed by Temudo et al., (2007), which showed that low pH values favored butyrate and acetate production, while higher pH values favored acetate and ethanol production, from glucose fermentation. Also, Jankowska et al., (2015) reported that with the increase of pH, the production of butyrate decrease due to the dominance of a facultative anaerobic bacteria which lacks the enzymes involved in the butyrate production.

The increase of acetate concentration with pH was also observed by Lim et al., (2008) (Table 3) which only tested three pH values, 5, 5.5 and 6, and achieved a higher acetate production at pH 6. The higher production of acetate at high pH values could be an advantage if the final application of SCOAs was denitrification. This, because acetate is the preferential acid consumed by denitrifying bacteria (Jiang et al., 2013). Though, for PHA production, it is preferential to enhance propionate production.

As can be seen, pH 6 favored propionate production, with 27.0%, when compared to pH 7 and pH 8, with 1.28% and 1.88%, respectively. However, when compared to the results of CSTR1 at HRT2, propionate percentage achieved at pH 6 was slighter below than the 38.3% achieved for the system without pH control. This was not expected since CSTR1 was operated at a pH lower than 5 during the whole fermentation time. Also, at pH 5, the results achieved for both AS and FS experiments were the lowest in terms of propionate production. Furthermore, despite it is reported that some propionate-producing bacteria have an optimal pH of 7-8 for cell growth (Horiuchi et al., 2002), in this work it was verified a major

diminution of propionate production for pH values above 6. Since the percentages of propionate achieved were not the expected, other methods could be used to enhance propionate production, like the addition of bicarbonate once it may favor propionate production (Temudo et al., 2008).

The results obtained in this work are not concordant with those obtained by Horiuchi et al., (2002), that tested pH from 5 to 8 and verified that from pH 5 to 7, acetate and butyrate were the main SCOA produced, with low concentrations of propionate. For pH 8, on the other hand, the main SCOA observed were acetate and propionate, with a decrease of butyrate concentration (Horiuchi et al., 2002). Despite that, these results support the fact that the production of propionate and butyrate have an inverse relation, which was already observed for CSTR1. Figure 17 shows this inverse relation observed for CSTR2.

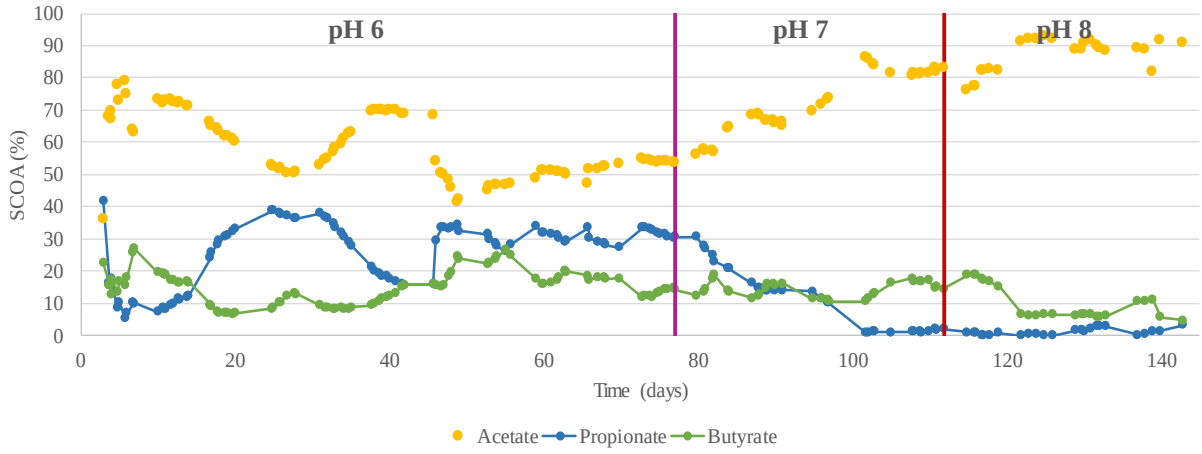

Figure 17. Representation of the acetate variation along with the inverse variation of propionate and butyrate, in % calculated from gCOD/L, in the CSTR2 for the three pH values tested along with the fermentation time.

The average SCOA profiles for pH 6, 7 and 8 were calculated considering the PSS. Once more, considering the order lactate, acetate, propionate, butyrate and valerate, the SCOA profiles achieved were 0.01/57.8/27.0/14.8/0.36 % for pH 6, 1.32/82.7/1.28/14.9/0.11 % for pH 7 and 1.55/89.2/1.88/7.20/0.26 % for pH 8. As can be seen, considering the deviation of carbon to the production of the other metabolite, possible succinate, pH 6 proved to be more efficient in terms of SCOA production and diversity, which was in accordance with the results achieve by Bengtsson et al., (2008) that proved that the optimum pH to achieve higher SCOA production was 5.5-6 also for a paper mill effluent. Plus, besides the

change in HRT in CSTR1 and along with pH 8, pH 6 was the value at which the system adapted more quickly (Figure 16).

4.4.4. Biomass variation

The variation of the biomass concentration is represented in Figure 18. After the inoculation, the biomass concentration suffered a major decrease, from the initial value, 11.6 g/L, to 2.47 g/L at the day 18, from which biomass values suffered variations between mainly 2.00-4.00 g/L. The decrease of biomass during the start of the fermentation was expected due to the acclimatization of biomass (Fernández-Morales et al., 2010) and was already observed by Queirós et al., (Submitted). It is known that in the first stages of acclimatization some organisms cannot adapt to the conditions imposed and have an inefficient metabolism (Rezouga et al., 2009). Thus, the stabilization of the biomass decrease after the inoculation was consistent with the achievement of the PSS, at day 17.

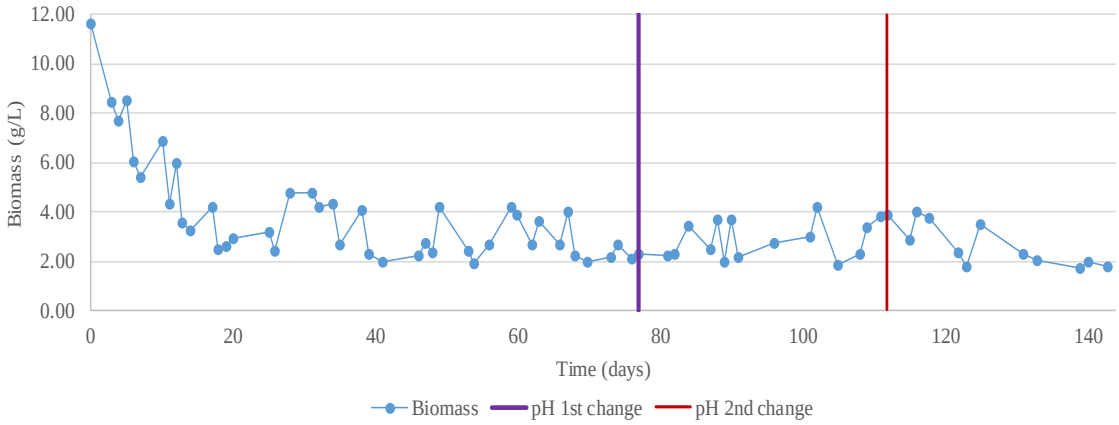

Figure 18. Variation of biomass concentration, in g/L, during the fermentation time in the CSTR2 for the three pH values tested.

The maximum biomass concentration achieved after the acclimatization was 4.80 g/L at the day 31 at pH 6 and the minimum was 1.74 g/L at the 139º day at pH 8. Despite the maximum and minimum values, the biomass concentration did not seem to have a relation with the system pH and remained quite stable for the three pH values tested. However, the values of biomass decreased slightly for pH 8.

4.5. MBBR

4.5.1. Choice of the reactor configuration and operational conditions

The use of a MBBR system allows to combine suspended growth with fixed film processes, making use of their best characteristics and advantages without being restrained by their disadvantages (Oliveira et al., 2014; Sheli et al., 2014). These systems have been used in the past few years in the treatment of effluents (Borkar et al., 2013; Oliveira et al., 2014; Sheli et al., 2014), but the study of AF using MBBR is limited. Working without pH control is advantageous since no reagents and further equipment were necessary, thus lowering the process costs. Such fact is crucial in the scale up of every processes. However, reactors that operate at low pH require a moderately long SRT because low pH values can inhibit the microbial growth due to the deviation of the use of energy to the maintenance of the intracellular pH. The intracellular pH is maintained by actively pumping out undissociated acids which diffuse over the cell membrane into the cell (Tamis et al., 2015). The use of long SRT is, then, essential and can be achieved by the introduction of a system for biomass retention. In addition to this, the low pH had a negative effect on the conversion of sugars into SCOA, as observed in CSTR1. The use of a reactor with a biomass retention system, such as MBBR, appeared as a way to avoid these problems.

In order to be able to compare the MBBR system performance with the CSTR1, the operational parameters imposed to the former were the same as those imposed to CSTR1, with a HRT of 3.01 days. In this way, an OLR of 5.95 gCOD/L·d was imposed with a flowrate of 0.66 L/d, at a temperature of 30 ºC. The inoculum used was fresh sludge that came from an aerobic tank with a concentration of 11.6 g/L.

The carriers chosen to perform the acidification of HSSL in the MBBR system were bioflow 9, due to its characteristics, namely diameter, low density and high specific surface area. Moreover, Sheli et al., (2014) compared the performances of bioflow 9 and bioflow 30 in the treatment of winery wastewaters and showed that the first one was more efficient in the attachment of biomass, which was associated to a higher specific surface area. The percentage of carriers used (41%) was chosen taking into account the literature (Arroja et al., 2012). Also, the fact that the pump could not be able to stir the medium containing the carriers was considered, since the medium was dense. Thus, despite some authors used

filling fractions between 60 and 70% of carriers for the treatment of wastewaters (Sheli et al., 2014; Sheli and Moletta, 2007; Wang et al., 2009a), in this work the decision of using a less percentage of carriers was made in order to avoid possible problems with the stirring system.

4.5.2. Results of the acidogenic fermentation of HSSL

The MBBR was operated for 47 days. The SCOA distribution and the SCOA, substrate and pH variations in the reactor are showed in Figure 19. In the first 26 days a major instability was observed in the system, with variations in SCOA concentrations along with the production of lactate. Such behavior, at low pH values and instability phases, was already seen in CSTR1 and in the batch experiments. Also in the first 26 days, the consumption of the sugars was not complete.

One of the main advantages of biofilm reactors is the reduced start-up time when compared to the conventional anaerobic systems (Escudié et al., 2011; Karadag et al., 2014). The MBBR needed 26 days while CSTR1 took 41 days to achieve PSS. Both MMC were taken from an aerobic tank, even if at different times, so this difference could be a result of the use of the biofilm system, that reduced the start-up time of the AF process, as expected. Other advantage of biofilm reactors is the capacity to tolerate high OLR, thus enhancing the productivity of the system (Escudié et al., 2011; Karadag et al., 2014), since more influent is converted for unit of time. Also, since the microorganisms are attached to a support, they show more resistance to organic load shocks, SCOA accumulation or even to inhibitors of the influent, which is important in the case of HSSL that contains many inhibitory compounds.

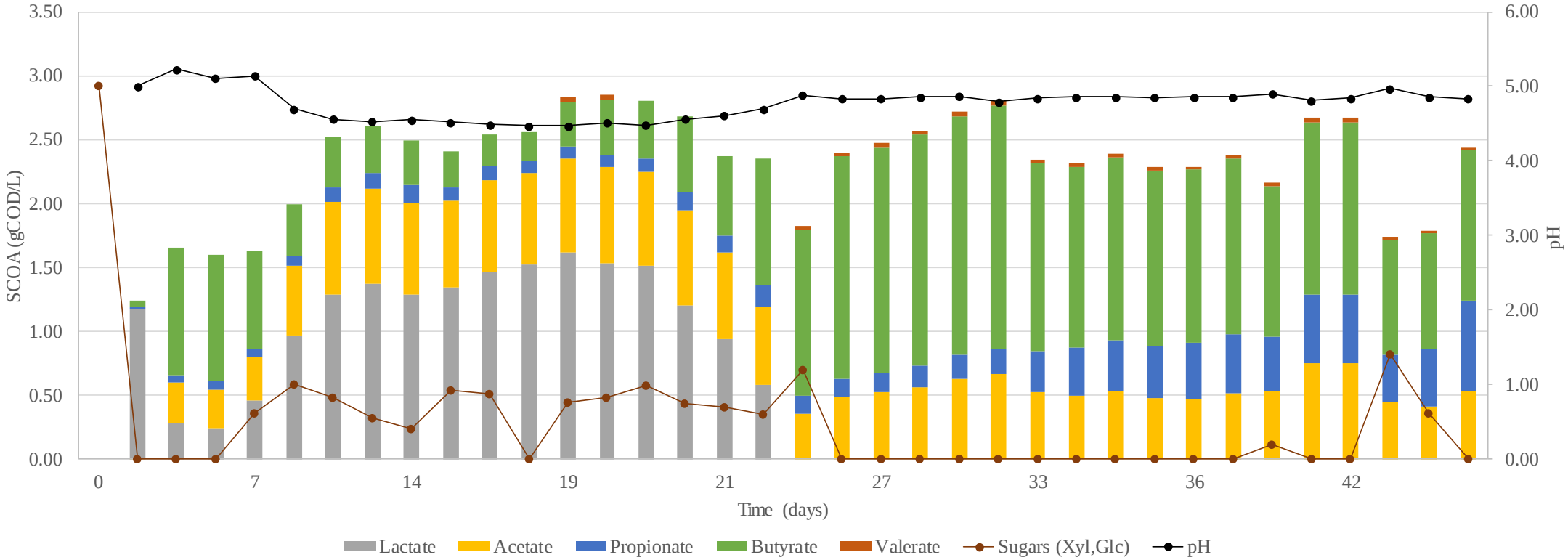

Figure 19. Distribution of SCOA and its variation, along with the substrate (xylose and glucose) and pH variation over the fermentation time for MBBR system.

The high efficiencies associated with MBBR systems are known to remain stable under higher OLR values applied, as stated by Karadag et al., (2014). The SCOA production remained stable during the PSS with exception for the days 43 and 44, that were associated with an increase of the sugars present, indicating that some perturbation of the system may have caused this drop in the SCOA concentration (Figure 19).

As CSTR1, in the MBBR a preferential consumption of glucose instead of xylose was observed. Xylose was only consumed when glucose was depleted. Contrary to CSTR1, this tendency was observed during the first instability period only. In the PSS this tendency could not be verified since both glucose and xylose were totally consumed, with exception for days 43 and 44 as stated earlier. Also, in the first 26 days the pH dropped from the initial value of 5.00 to 4.47 in the 19º day. When the pH values remained between 4.8 and 4.9, the system could be considered stabilized, which, once more, proved the major impact of pH in the system stability.

The maximum SCOA produced in MBBR was 2.85 gCOD/L, less than half of the value achieved in CSTR1, but higher than the values obtained for CSTR2 for pH 7 and 8 (Table 6). Considering the yields, a value of 0.86 gCOD/gCOD was achieved for $Y_{SCOA/Sugars}$ in MBBR which was lower than the values achieved for CSTR1 at HRT2, 1.35 gCOD/gCOD. However, it was higher than the values obtained for CSTR2 for the three pH values tested. Considering $Y_{SCOA/S}$, a value of 0.73 gCOD/gCOD was obtained, similar to those achieved for CSTR2 for all the pH values, and lower than the 0.89 gCOD/gCOD achieved for CSTR1 at HRT2. The average AD_{Sugars} achieved in MBBR was 79.5 %, lower than the values calculated for CSTR1 and CSTR2 for pH 6 and 7. The AD_{total} was 13.0 % and the comparison with the other systems tested was the same as for AD_{Sugars}. The maximum AD_{total} obtained was 16.0 % at the day 20 and corresponded to the maximum SCOA concentration achieved. Just like in CSTR1, these maximum values were achieved before the PSS.

4.5.3. SCOA distribution

The SCOA profiles obtained were 23.0/23.7/10.0/42.6/0.72 %, for the whole fermentation time, and 0.00/23.0/15.9/59.9/1.21 %, for the PSS, for lactate, acetate, propionate, butyrate and valerate, respectively. Noteworthy the difference in the lactate percentages for the two profiles presented. The acetate distribution remained quite stable

during the whole process. The main differences were actually in the propionate and butyrate concentrations, that achieved major concentrations in the MBBR when PSS was reached.

In MBBR system, the SCOA distribution varied throughout the fermentation time until the last day, including during the PSS. This variation was verified especially in the propionic and butyric concentrations, proving again the shift between these two SCOA, observed mainly in the PSS. Despite butyric acid was the main SCOA during the whole PSS state, at the end of the fermentation time the propionate concentration was higher than in the beginning of PSS. There was no time to test the system above the 47 days, however it would be interesting in order to ensure a constant SCOA distribution, thus making the effluent suitable to be used in the applications referred earlier.

Also, it is important to note that along with CSTR1 at HRT2, this was the only period during which acetate was not the main acid produced. In the MBBR, high butyrate concentrations were observed even at the end of the fermentation time in which the propionate concentration was higher. The higher butyrate concentrations observed in MBBR when compared to CSTR1 could be associated with the pH value, that was slightly lower in the CSTR1, with pH varying from 4.3 to 4.6 while in this case varied from 4.8 to 4.9, considering only the PSS for both cases. Thus, higher butyrate production may be associated with the competition between the propionic and butyric producer bacteria, as already stated previously.

The production of valeric acid during the PSS remained quite stable when compared to the other systems tested, even if produced at low concentrations, representing 1.21 %, in average of the total SCOA produced. The fact that this SCOA started to being produced after the lactate disappearance is in accordance with what was previously discussed for CSTR1, where the suggestion of the consumption of lactate to produce other SCOA, including valerate in small amounts, was proposed.

4.5.4. Biomass variation

The variation of the biomass in the MBBR is represented in Figure 20. As observed in the CSTR2, after the inoculation the biomass concentration decreased from the initial 11.6 g/L to 3.38 g/L at day 9. After this day, the values varied slightly for the rest of the fermentation time. The variation in biomass could be related not only with the start-up strategy used to the acclimatization and attachment of the MMC to the carriers, but also to

the small variations suffered by the system during the operation time. The drop in biomass concentration related to acclimatization in the beginning of the process was not seen in CSTR1 since in this case the acidogenic populations were already selected. Despite this, when compared to CSTR1 and CSTR2, the biomass concentration in the MBBR was more stable, especially after the PSS was reached. This may be associated with the constant attach/detachment of the biomass to the carriers, a normal process in a hybrid system like this, containing two different types of growth (Qiqi et al., 2012).

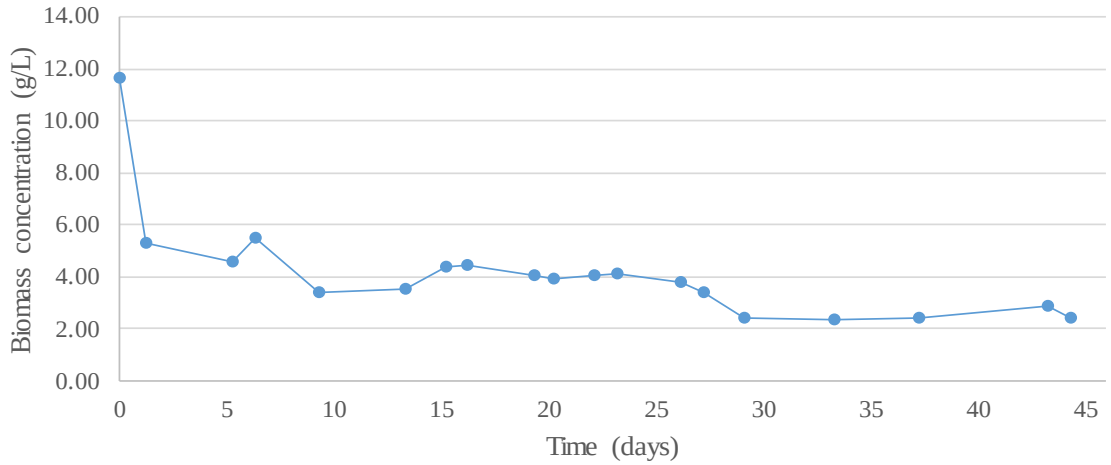

Figure 20. Variation of biomass concentration, in g/L, during the fermentation time in the MBBR.

The maximum biomass concentration achieved after the acclimatization was 4.43 g/L for day 16 and the minimum was 2.33 g/L for day 35. These values were similar to those obtained for CSTR1 and CSTR2. Such fact is crucial, since besides biomass attached to the carriers, a considerable concentration of biomass remains in suspension, allowing higher capacity of degradation of carbon compounds (Oliveira et al., 2014).

The volatile attached solids concentration was not evaluated in this work. Once again, it is the important an evaluation of the microbial communities present in the system and also attached to the carriers. The comparison of the microbial populations changes through acclimatization for CSTR2 and MBBR would also be interesting, just like the assessment of the biofilm formation process.

5. Concluding Remarks

During the present study, different factors were tested for the AF of HSSL. First, a CSTR1 system was operated without pH control and at two different HRTs . Then, the influence of the initial pH on the SCOA distribution and acidification performance was tested in the pH batch experiments. Taking into account these experiments, a CSTR2 system was tested, and the results were compared to those obtained in CSTR1. Lastly, another reactor conformation evolving both suspended and attached growth, a MBBR system, was tested and compared with the conventional CSTR systems.

In general, for all the experiments carried out, the main SCOA produced were acetate, propionate, butyrate and in minor amounts, lactate and valerate. The production of lactate was associated not only with the lower pH values verified in the system, but also with instability and adaptation phases of the fermentation. Sugars consumption showed to have a relation with the pH of the system, with the higher pH values favoring the sugars degradation. Furthermore, the glucose was consumed preferentially to xylose, fact associated with the metabolism of conversion of the two sugars. A shift between propionic and butyric acids was observed in all the systems tested and was associated with the dominance of propionic and butyric bacteria. Furthermore, pH showed a major importance in the stability of the process and in the SCOA profiles achieved.

In CSTR1, AD_{sugars} higher than 100% were achieved, indicating that other monomeric sugars present in HSSL were being consumed. The average SCOA concentrations achieved for this reactor were 3.10 and 3.53 gCOD/L for HRT1 and HRT2, respectively. For PSS1, acetate was the main SCOA produced with 36.7%, whereas for PSS2 propionate was dominant with 38.3%. Considering the effluent characteristics, the SCOA profile achieved was 3.57/53.5/23.7/19.3/0.62 % for HRT1 and 2.50/44.5/30.1/20.4/2.14 % for HRT2, considering the order lactate, acetate, propionate, butyrate and valerate. The high propionate percentages obtained constitute an advantage for the downstream process applied in this case – the PHA production. LS were not consumed.

The pH experiments showed that FS and pH 5 were the most efficient in terms of SCOA production, with 9.11 gCOD/L. For the experiments, an AD_{sugars} higher than 100% was also observed. For both FS and AS tests, pH 4 revealed the poorest results for acidification, being then considered an inhibitory value for the MMC. Since for the highest

pH values the buffer solution did not work, besides pH 6, also pH 7 and pH 8 were chosen to be tested in CSTR2. For these batch experiments, LS were not consumed, just like in CSTR1. This fact allows to conclude that these recalcitrant compounds of HSSL were not consumed, even using a fermentation time of more than 20 days. Thus, one of the main goals of this study was not achieved. On the other hand, since LS were not consumed, a step of LS extraction before AF must be considered and should be advantageous to the overall process, since it could increase the variety of products achieved and enhance the potential of the acidification process.

The CSTR2 system showed a higher stability when compared to CSTR1, but lower SCOA average values, with 2.36 gCOD/L for pH 6, 2.38 gCOD/L for pH 7 and 2.27 gCOD/L for pH 8. The results for pH 6 were not expected, due to the batch experiments results, but can be explained by the production of other metabolite in major amounts that can possibly be succinate. Again, the effect of pH on the SCOA profiles was proved. Contrary to CSTR1, in this case the sugars were totally consumed.

The MBBR was tested in this work and an average SCOA concentration of 2.32 gCOD/L was achieved, lower than those obtained for CSTR1. On the other hand, this system revealed a shorter start-up time when compared to CSTR1. The profile achieved was 0.00/23.0/15.9/59.9/1.21 %, showing that this reactor conformation had as main SCOA produced butyrate. Despite butyrate was the main SCOA produced, propionate concentrations increased during the PSS, which indicate that possibly the dominance of certain species may be responsible for this shift.

For all the experiments realized, the AF was proven to be an efficient process which not only produced value-added chemicals, SCOA, but also allowed the valorization of an organic-rich stream, the HSSL.

6. Future Prospects

Despite the conclusions reached in this work, further work goes through the evaluation and testing of the different parameters evolved in the AF process, and also of the different reactor conformations that can be used for that purpose.

Higher pH values were not tested in the CSTR2 due to lack of time. It is reported by some authors that high pH values could have an inhibitory effect on the fermentation since the acidogens generally function from pH 4 to 8.5 (Appels et al., 2008; Maspolim et al., 2015). Nevertheless, higher pH values should be tested in the future because other authors defend that the optimal pH values for the production of SCOA are in the range of 5.25-11 depending on the type of carbon source used, which also have a major impact on the success and behavior of the process (Lee et al., 2014). Hence, the behavior of acidogenic microorganisms varies accordingly with the substrate used and it would be interesting to evaluate in the future the effect of pH higher than 8 in the AF of HSSL. Also, for CSTR2 the sugars were all consumed in the three pH values tested for almost all the fermentation time, thus further work goes through the testing of an OLR increase in order to evaluate the behavior of the system and how the increase of this parameter could enhance the yield of the process.

The fact that the AD_{sugars} achieved were higher than 100% make the quantification of the other monomeric sugars present in the HSSL an urgent step to be done in a near future to allow a better understanding of the real use of the carbon source. Besides, the study of other pH values in a system like CSTR2 should be done in order to stablish a complete relation between pH and SCOA profiles and concentrations. This will allow to have a better control of the process in the future and to enhance the SCOA profiles considering the application of the effluent. Also the identity of the unknown compound produced in CSTR2 at pH 6 needs to be investigated. This is crucial since the compound produced deviated major amounts of the carbon present in the stream and consequently lower the SCOA average concentration and respectively the AD.

Also, the MBBR system must be studied in more detail, which includes in the first place an elongation of the fermentation time to stabilize the profiles achieved. Next, it would be essential to study the influence of parameters such as pH, OLR, HRT and temperature. A first step should be the introduction of a pH control system in the MBBR using the same

conditions of this work. The comparison between a CSTR and MBBR systems with pH control at various pH values is crucial and must be done near in the future. Then, taking into account the results achieved for MBBR in the present study and considering the literature regarding high organic loads (Escudié et al., 2011), the testing of higher OLR appears to be a crucial step in order to enhance the potentialities of this type of system. This way, it will be possible to study and optimize the AF process in another reactor configuration besides CSTR, since it shows such advantages. Also, these tests must be carried out for different streams in order to evaluate the potential of each one in acidification.

It is important to note that the biomass concentrations presented in the current study only inform about the risk of washout and the stability of the whole culture though parameter variations. Moreover the study of microbial composition has revealed crucial to understand the mixed culture processes. Thus, in the future is important to focus in the in the microbial diversity analysis and in the identification of the dominant metabolic groups of the process (Temudo et al., 2008). Also, the impact that pH has on the microbial communities and thus, on the product spectrum and how these microorganisms react to operational changes must be studied carefully and separately for each substrate used, and in this case, for HSSL. This is relevant since it will allow in the future to enhance the SCOA production and achieve the required SCOA profiles..

7. References

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8. Appendices

8.1. Appendix A: AS Batch Experiments

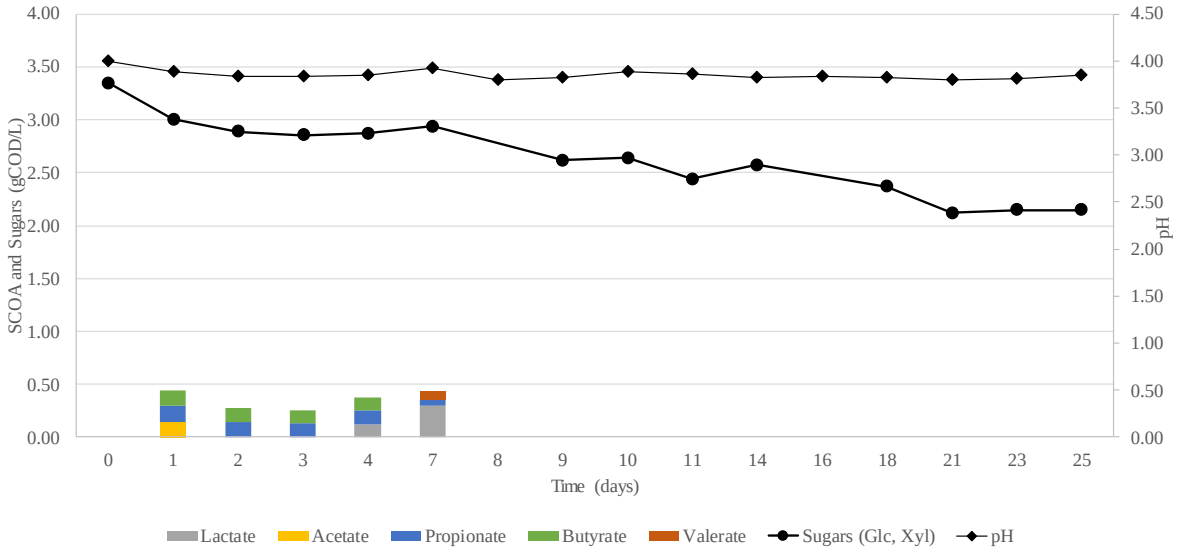

Figure 21. AS batch experiments – pH 4.

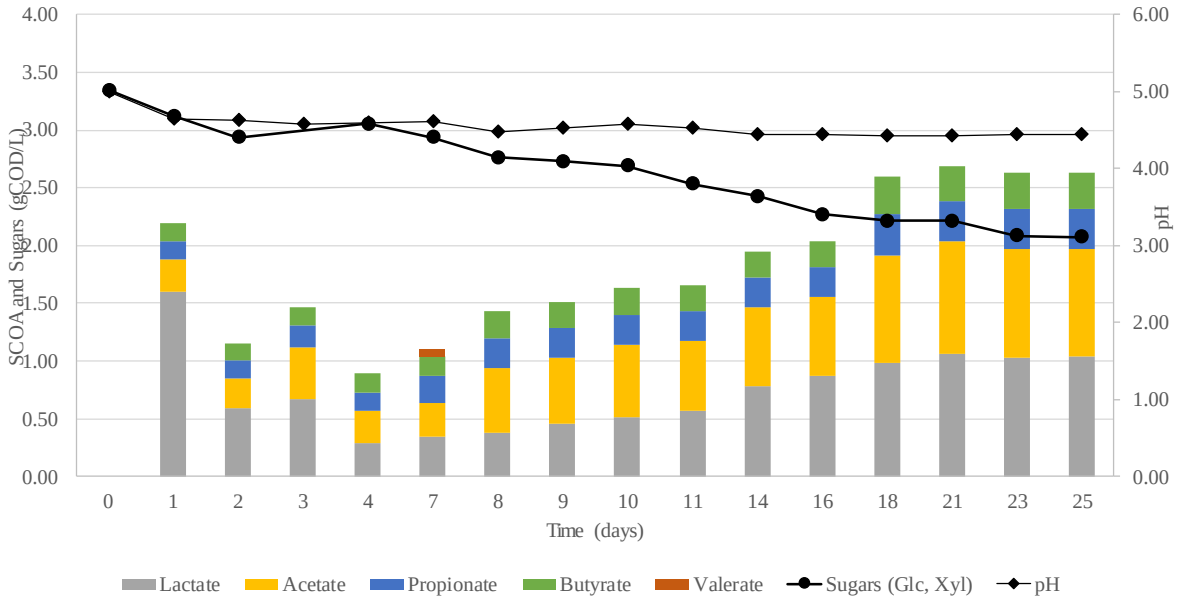

Figure 22. AS batch experiments – pH 5.

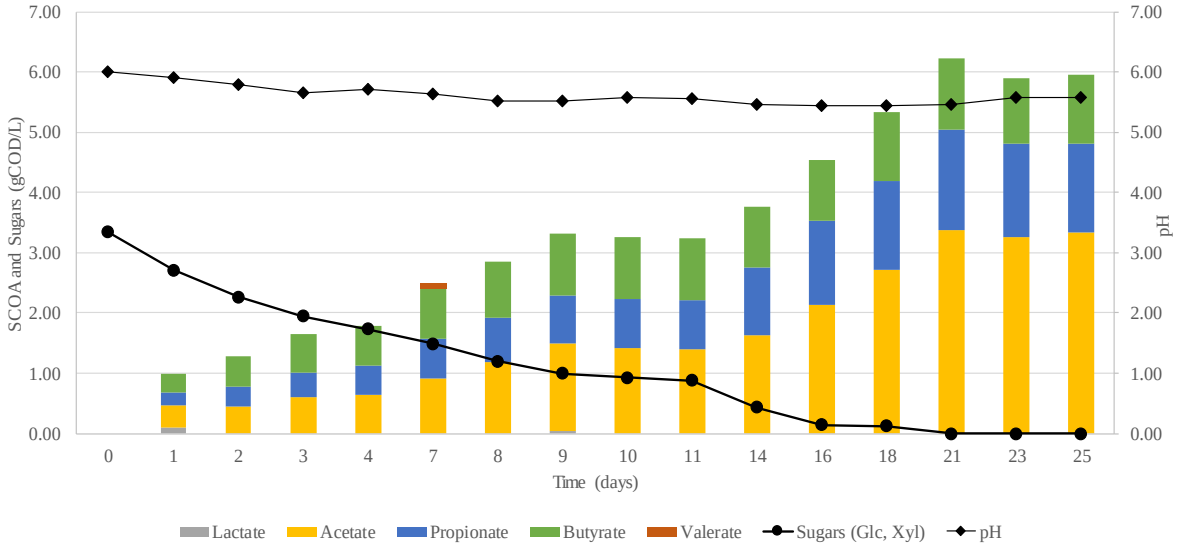

Figure 23. AS batch experiments – pH 6.

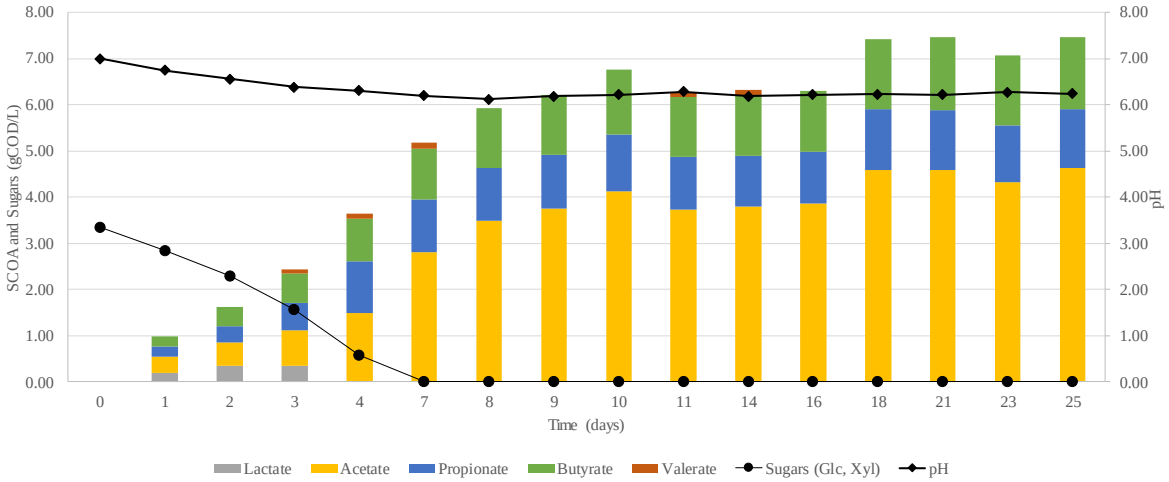

Figure 24. AS batch experiments – pH 7.

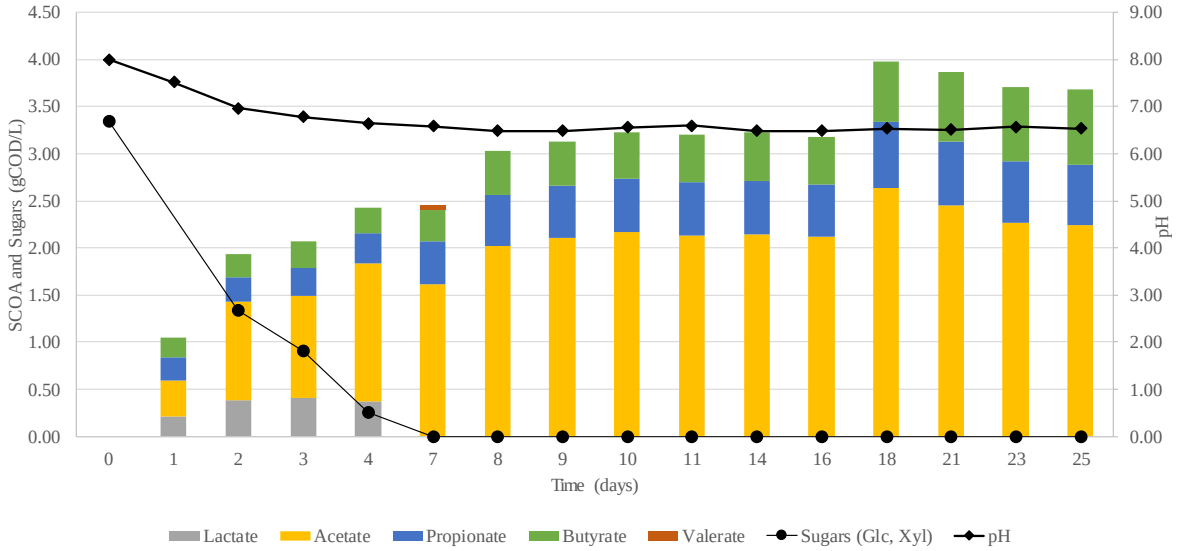

Figure 25. AS batch experiments – pH 8

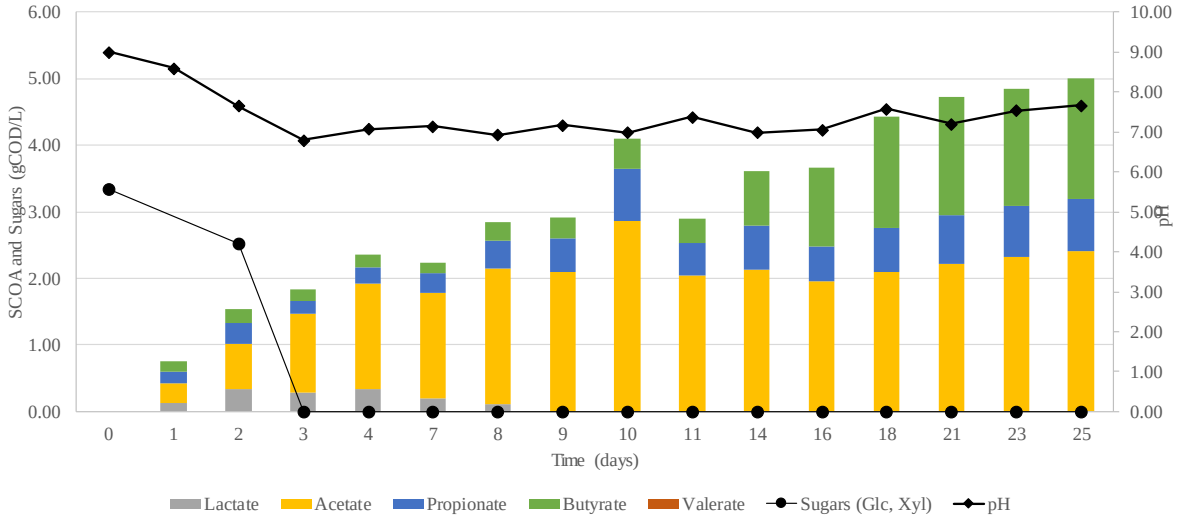

Figure 26. AS batch experiments – pH 9.

8.2. Appendix B: FS Batch Experiments

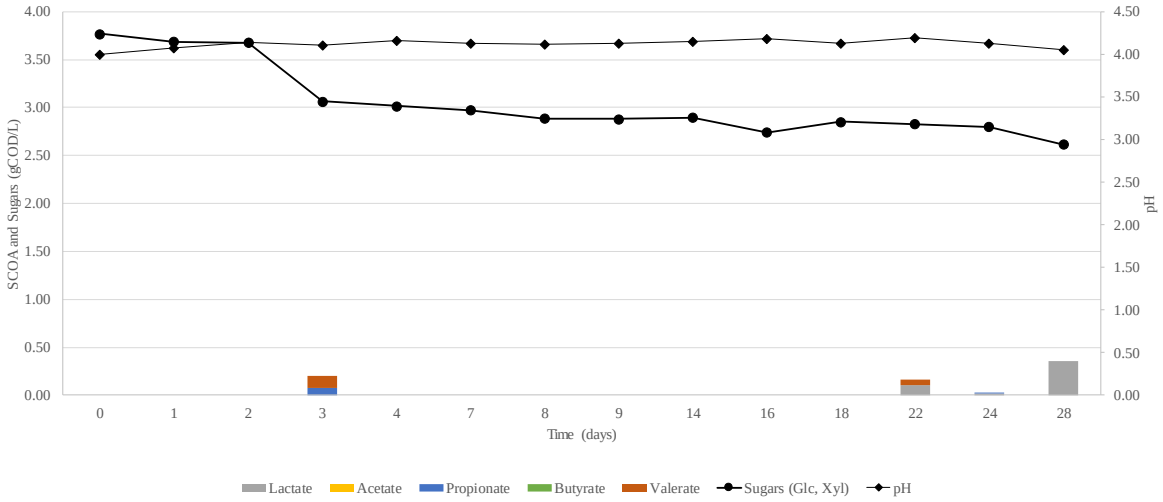

Figure 27. FS batch experiments – pH 4.

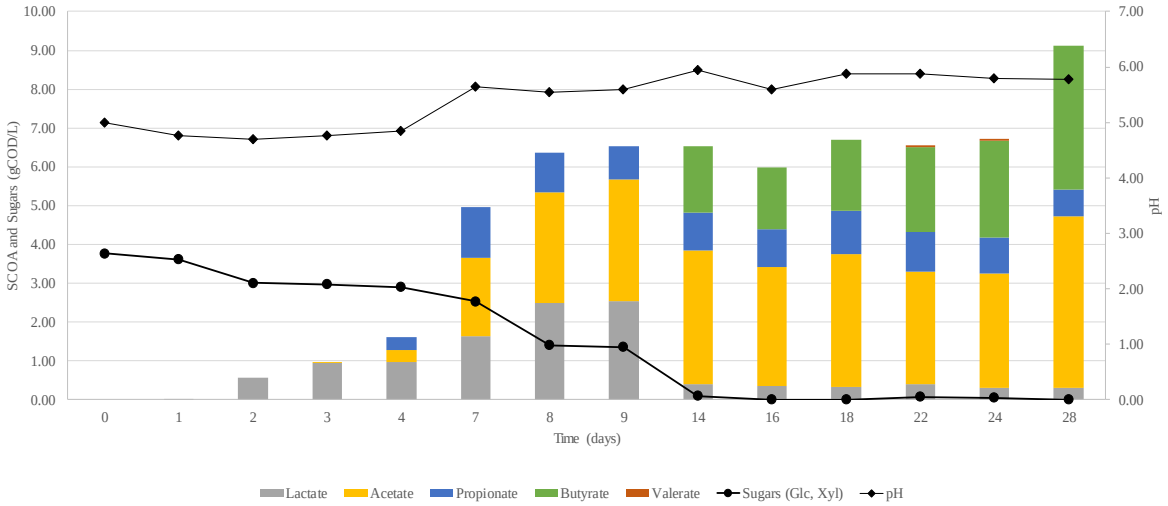

Figure 28. FS batch experiments – pH 5.

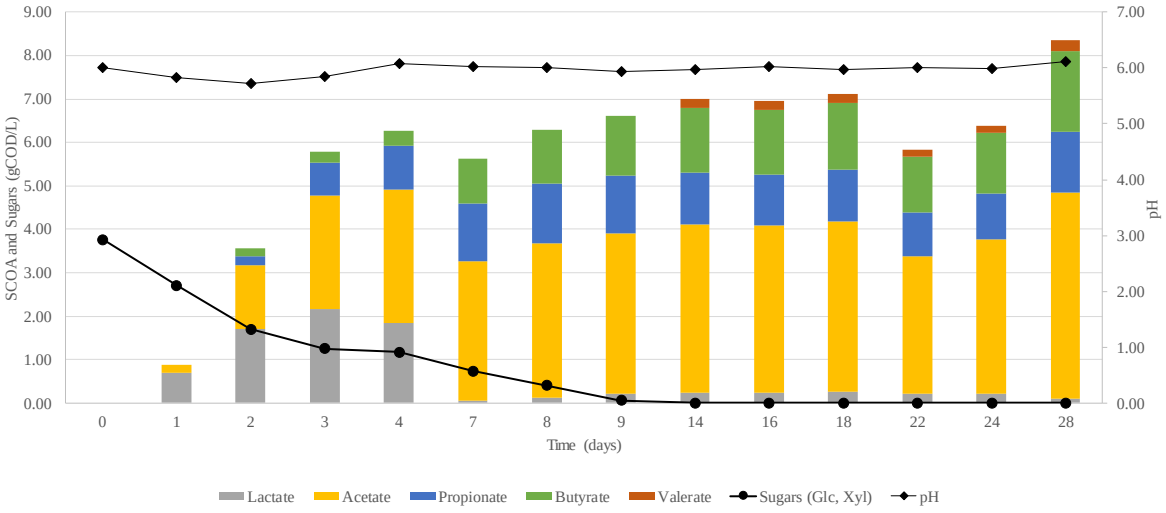

Figure 29. FS batch experiments – pH 6.

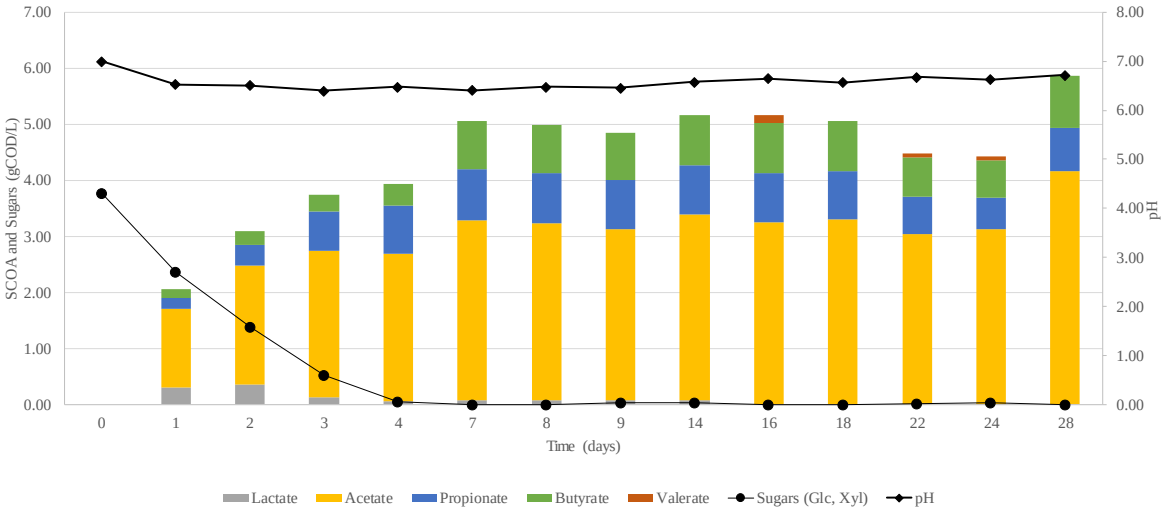

Figure 30. FS batch experiments – pH 7.

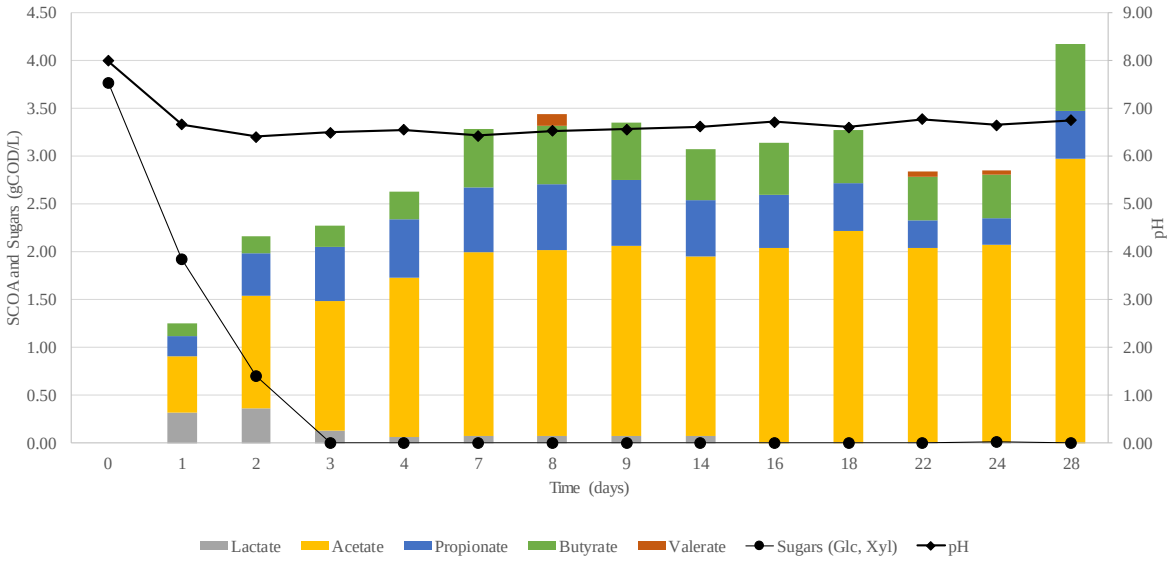

Figure 31. FS batch experiments – pH 8.

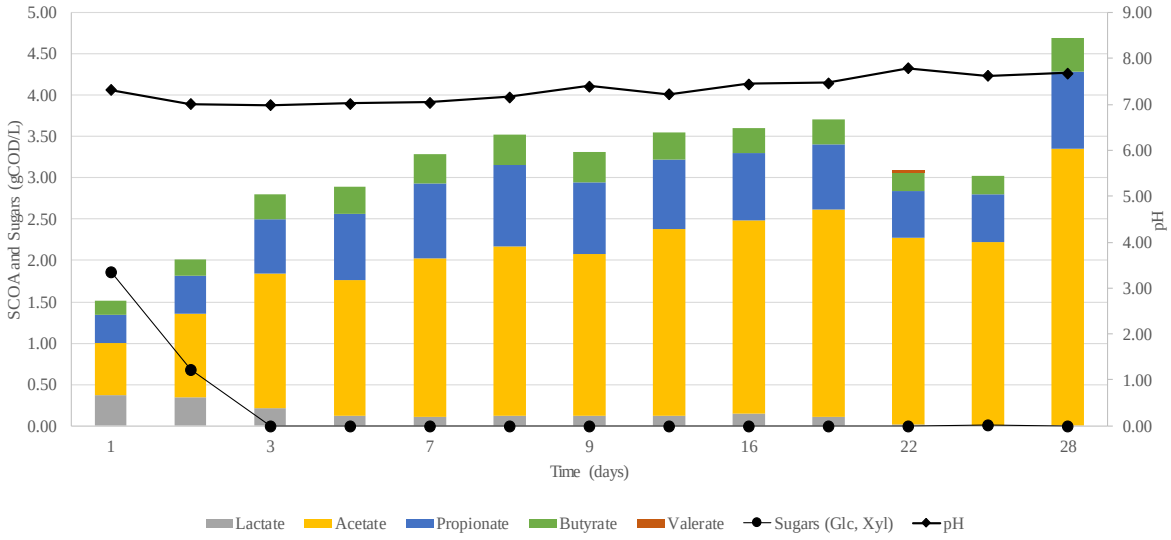

Figure 32. FS batch experiments – pH 9.